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Obituary Notice

WALTER RAMSDEN, 1868–1947

Walter Ramsden, whose death was announced on 27 March 1947, was an original member of the Biochemical Society. His loss will be felt keenly by his many friends, both on account of his amiable personality and of his scientific interests.

The fourth son of W. H. F. Ramsden, a physician of Saddleworth, Yorkshire, and of Catherine Kay, he was born on 4 October, 1868. At the age of 14, owing to trouble with his eyes, he left Manchester Grammar School, which he had entered when twelve years old, to study privately for four years at home. He came to Oxford with an Exhibition at Keble College, and obtained a 1st Class in the Honour School of Physiology in 1892. He was Radcliffe Travelling Fellow from 1893 to 1896, and during this time he studied at Zürich and Vienna and finally at Guy's Hospital, taking his Final M.B., B.Ch. in 1897 and his M.D. in 1902. Never married and never in medical practice, he devoted himself to teaching and research.

In 1899 he was elected at Pembroke College to the Sheppard Medical Fellowship which he held to the end of his life. It is interesting to note that under the old statutes this Fellowship is tenable for life unless forfeited by marriage. His talents were soon utilized in the Department of Physiology where he became Lecturer in Physiological Chemistry under Prof. Gotch. He held the post until 1914, and during his last year, he worked under Prof. Sherrington who had succeeded Gotch. He took part in other University activities and exercised a stimulating influence on his pupils; Edward Whitley, for instance, later became with Benjamin Moore the joint founder of the *Biochemical Journal*, and subsequently started Biochemistry in Oxford with the endowment of the Whitley Chair, Moore being the first holder.

From Oxford Ramsden went in 1914 to Liverpool to become Johnston Professor of Biochemistry in succession to Benjamin Moore. The effect made in Liverpool by his personal charm is well described in a recent obituary notice (*Nature, Lond.*, 1947, **159**, 801).

In 1931 he retired to devote his remaining years to research in Oxford. For the first few years he worked in the Department of Pathology and from 1937 in the Department of Biochemistry. He pursued his researches on proteins from the silkworm until the end, so far as failing health and a troublesome gastric condition would permit. For many years he had not enjoyed good health, but nothing interfered with the charm of his old world courtesy. His ready reception of new ideas and willingness to discuss them remained as characteristic as his fund of anecdotes. Many will retain the vivid impression of his appearance in the appropriate season with a tin containing a kindly gift of mulberries.

Those who knew him early in his career, and appreciated his brilliant skill as an experimenter in the field of surface chemistry, were disappointed that his early promise did not materialize with sufficient solidity in print. Though he was ever willing to discuss his work and ideas with friends, a certain fastidiousness and a wish to obtain more thorough completion, inhibited the published word. Nevertheless the work which appeared was important. He was among the earlier biochemists to appreciate the importance of physico-chemical surface studies in biology. His first researches set the stage for his life's devotion to the phenomena of surfaces in their especial relation to protein chemistry; in 'Die Coagulierung von Eiweisskörpern auf mech. Weg' (*Arch. Anat. Physiol., Lpz.*, 1894, pp. 517–34), he showed how denaturation of proteins occurred at the interfaces formed by mechanical shaking; this was to be followed later by work on the 'separation of solids in surface layers of solutions' (*Proc. Roy. Soc.* B, 1903, **72**, 156; also *Z. phys. Chem.* 1904, **47**, 336). The latter work is not as well known as it should be and has a pioneer relationship to some modern protein film work. In 1902 (*Proc. Physiol. Soc.* 5 July) he described some new properties of urea; proteins were dissolved by urea with denaturation; coagulated proteins went into solution, and even a 'dead frog placed in saturated urea solution becomes translucent and falls to pieces in a few hours'; 'thiourea appears to have precisely the same effects as urea'.

This pioneer work paved the way for more recent investigations with urea and guanidine acting on proteins. With N. G. Chavasse, in the effect on viscosities of denatured egg albumin solutions, it was followed in detail and formed a contribution to a Faraday Society Discussion in March 1913 (also *Z. Chem. Industr. Kolloide*, 1913, **12**, 250), and further details were published with J. Hatton (*Chem. and Ind.* 1930, **8**, 851); Ramsden showed, for instance, that urea unmasked the thiol and disulphide groups in egg albumin and added details upon the rate of denaturation. During World War I, he worked with colleagues upon the estimation of quinine (see Ramsden, *Brit. med. J.*

1920, **2**, 117; papers in *Ann. trop. Med. Parasit.* **11**, **12** and **13**). This investigation occupied much time and the details proved troublesome. After this interlude, he soon returned to surfaces, and a more fully developed account of his theories was given in 1923 (Presidential address on Surfaces and Films to the Liverpool Biological Society; 'Demonstrations in Interfacial Phenomena at British Association Meeting', *Nature, Lond.*, 1923, **112**, 671).

There followed in 1926 (*Trans. Faraday Soc.* **22**, 484) a fuller account of part of his theories in a discussion ('Some Physical Properties of Composite Surfaces'), embodying work carried out with R. H. Hurst (see also *J. Soc. Chem. Ind., Lond.*, 1929, **15**, 1113). In particular, it was noticed that some insoluble liquids or water-soluble colloids (saponin), would spread to enormous areas upon pure water surfaces. This led to his conception of 'disturbance haloes', and to his insistence upon the reality of surface tensions. He always argued that it was erroneous to think Langmuir's apparatus measured a 'surface pressure' of the film particles upon the movable barrier, and preferred to consider them as produced by decrements in the respective tensions. This he held very strongly and hated the thought that there should be no reality in surface tension. In 1927, Ramsden described in Clayton's 'Theory of Emulsions and their technical treatment' his theory of emulsions stabilized by solid particles, in which characteristically he stated that with J. Brooks, he had devoted two years from 1922 to 1924 to an experimental test by numerous observations of the contact angle made against a variety of solids at water-benzene or paraffin interfaces. To the writer's knowledge, these data have never been published, though some of the complicated solubility relations holding with cholesterol for mixtures of kephalin and lecithin in systems containing water and a fat solvent were briefly described (Brooks, J., Ramsden, W. & Earlam, L. (1929), *J. Soc. Chem. Ind., Lond.*, **15**, 1113).

About 1930 there followed an introduction to work which occupied him in his retirement. A deeper investigation of the reactions given by sheep's wool, treated with aqueous urea solutions, led to the finding of a pseudo thiol reaction given with an alkaline urea solution and an 'aged' nitroprusside solution, and to some qualification of a previous statement. It is possible that this work upon wool led to the prolonged researches upon fibroinogen; how the stiff water-soluble fibroinogen in the silk depots (larva of *Bombyx mori*) is transformed into the insoluble fibroin strands of silk (*Nature, Lond.*, 1938, **142**, 1120).

Briefly the interesting new fact emerged that the protein fibroinogen, which is thermostable, is coagulated by shearing between two flat plates of glass, and by freezing. The presence of certain salts prevents the latter change. Interestingly enough, this coagulation by shearing was not a case of surface coagulation similar to that described by himself over 40 years earlier. Other details about this phenomenon were added later, at a Biochemical Society Meeting in Oxford (26 October) during World War II (*J. Soc. Chem. Ind., Lond.*, 1940, **59**, 730). At the time of his death, he was trying to confirm an important suggestion that a catalytic factor might be concerned.

This brief account serves to illustrate a life-long interest, but cannot describe at all adequately the influence of his earnest spirit of inquiry, and his refusal to let ill-health divert him from his interests. He had many hobbies; he was a strong advocate of Esperanto and was active in spreading that gospel. In spite of his poor health, up to within four months of his decease, he would cheerfully ride 20 to 30 miles in hilly country on his bicycle, and it was remarkable how easily he negotiated the now heavily congested roads between the Parks and Pembroke College.

(The writer gratefully acknowledges help from Sir Charles Sherrington and Mr J. R. P. O'Brien in the preparation of this account.)

R. A. PETERS

Studies in the Biochemistry of Micro-organisms

76. MYCELIANAMIDE, $C_{22}H_{28}O_5N_2$, A METABOLIC PRODUCT OF *PENICILLIUM GRISEO-FULVUM* DIERCKX. PART 1. PREPARATION, PROPERTIES, AND BREAKDOWN PRODUCTS

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(*Received* 5 *August* 1947)

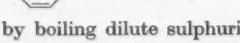
The occurrence of a crystalline, ether-soluble, nitrogenous compound in the mycelium of *Penicillium griseo-fulvum* Dierckx, grown on synthetic media, has previously been mentioned in several studies from this laboratory on the metabolic products of this mould. The compound was first encountered by Anslow & Raistrick (1931) in their work on the isolation of 6-methylsalicylic acid. It was mentioned again by Oxford, Raistrick & Simonart (1935) in their memoir on fulvic acid, and the same authors later (1939) devised a method for separating it from the accompanying griseofulvin. To extend the account of the synthetical achievements of this biochemically remarkable mould we now give a description of our researches, as far as they have gone, into the constitution of this nitrogenous product which we propose to name '*mycelianamide*', meaning an amide found in mycelium.

Mycelianamide, m.p. 170–172° decomp., is a colourless, laevorotatory, crystalline substance of empirical formula $C_{22}H_{28}O_5N_2$. It is sparingly soluble in the usual organic solvents and is very readily split both by acids and alkalis. The following is a list of the relevant facts concerning its properties and breakdown products.

(1) The compound has no basic properties but is a very weak acid, soluble in sodium carbonate solution but not in sodium bicarbonate or baryta water. It is at least a monobasic acid, but does not titrate sharply and suffers some decomposition during the titration. It has no phenolic properties. It could not be induced to give any reaction for a $>CO$ group.

(2) It gives an intense reddish brown colour with ferric chloride in ethanolic solution.

(3) After hydrolysis by hot concentrated HCl two bases were isolated as hydrochlorides, viz. ammonia and the known compound ω-amino-*p*-hydroxyacetophenone (1).

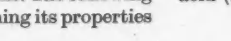
(1) 

(4) Hydrolysis by boiling dilute sulphuric acid yielded an inseparable mixture of non-volatile products. The volatile products included CO_2 (1 mol. if the hydrolysis was continued long enough), ammonia (1 mol.) and a sweet-smelling unsaturated hydrocarbon $C_{10}H_{16}$ (b.p. 177°) subsequently referred to as 'mycelene', which appears not to be identical with any known hydrocarbon of this empirical formula. Some observations concerning its constitution and relation to mycelianamide appear in a subsequent paragraph. A little acetaldehyde is also produced in this hydrolysis.

(5) Hydrolysis by boiling dilute sodium hydroxide or sodium carbonate (or even bicarbonate) yielded ammonia (approximately 1 mol.) and an optically inactive, monobasic acid $C_{17}H_{22}O_3$, m.p. 118–120°, which has no phenolic, alcoholic or ketonic properties. On further hydrolysis with dilute sulphuric acid it yielded mycelene and *p*-hydroxybenzoic acid ($C_7H_6O_3$). This decomposition can be represented very simply as follows:

$$C_{17}H_{22}O_3 = C_{10}H_{16} + C_7H_6O_3,$$

and the acid $C_{17}H_{22}O_3$ is clearly *p*-myceloxybenzoic acid (2; $R = OH$),

(2) 

the third oxygen atom possessing an ether function. It is reasonable to assume that mycelene arises from a hypothetical alcohol, mycelyl alcohol $C_{10}H_{17}OH$, by loss of water, and that the cause of the remarkable instability of the ether link in the acid $C_{17}H_{22}O_3$ must lie in the constitution of the mycelyl radical (see later).

(6) Hydrolysis by cold very dilute ammonia (0·03N), continued for several days at room temperature, yielded a neutral compound $C_{17}H_{23}O_3N$, m.p. 120°, which by further acid hydrolysis again yielded mycelene and *p*-hydroxybenzoic acid. This neutral compound is therefore p-*myceloxybenzamide* (2; $R = NH_2$). It is obviously of importance to find out if the N atom in this breakdown product arises from the ammonia used in the hydrolysis or whether it is an integral part of the molecule of mycelianamide. All attempts to obtain *p*-myceloxybenzamide from mycelianamide directly by the use of other

alkalis in dilute solution failed. These included NaOH (0·01N and 0·03N), methylamine, potassium carbonate, and various mixtures of potassium carbonate and sodium bicarbonate all acting in the cold at a concentration of 0·03N. Only with methylamine was an appreciable amount of an insoluble, neutral compound obtained, and this proved to be uncrystallizable. It must be concluded that there is no evidence at the moment against the view that the N atom of *p*-myceloxybenzamide is not an integral part of the mycelianamide molecule but originates in the ammonia used in the particular mode of hydrolysis which leads to its formation.

p-Myceloxybenzamide, as obtained above, is optically inactive. Since it is almost inconceivable that racemization could occur under the particularly mild conditions of this hydrolysis the important consequence follows that the mycelyl radical does not contain an asymmetric C atom.

(7) It is an interesting fact, although not of immediate importance with respect to the structure of mycelianamide, that this mould product exerts a marked bacteriostatic action on *Staphylococcus aureus*, quantitatively almost identical with that of 4:4′-diamidino-$\alpha\gamma$-diphenoxypropane (Ashley, Barber, Ewins, Newberry & Self, 1942).

As the simplest partial structural formula which will explain the above facts we suggest (3), viz. the *amide* of O-*mycelyl*-N-*pyruvyl*-β-*keto-tyrosine*, a structure which is obviously plausible from a phytochemical point of view.

(3) $C_{10}H_{17}.O$—C$_6$H$_4$—$CO.\overset{*}{C}H.NH.CO.CO.CH_3$.
$CO.NH_2$

Accepting the view previously arrived at that the $C_{10}H_{17}$ radical does not contain an asymmetric C atom it will be seen that (3) contains one asymmetric C atom, marked with an asterisk. The fact that mycelianamide is optically active eliminates one structure which is otherwise as satisfactory as (3), viz. that in which the side chain is

$$.CO.CH_2.NH.CO.CO.CH_2.CONH_2.$$

An attempt will now be made to account for the known facts listed above, concerning mycelianamide on the basis of (3).

(1) (3) contains no basic N atom, nor a COOH group. It ought, however, to be weakly acidic by virtue of the grouping .CO.CH.CO., which is known to confer acidity on benzoylacetic ester and related compounds.

(2) The presence of this same grouping will explain the reddish brown colour with ferric chloride.

(3 and 4) Acid hydrolysis may be supposed to remove the mycelyl radical leaving a phenolic OH, to convert the acid amide grouping to COOH, and also to split the NH.CO link. The resulting amino derivative of hydroxybenzoylacetic acid would readily lose CO_2 on further acid hydrolysis to yield structure (1). The acetaldehyde, which is produced in very small yield, might arise from the acetyl group at the end of the side-chain in (3) by the following side-reaction:

$$R.CO.CO.CH_3 + H_2O = R.COOH + CH_3.CHO.$$

(5 and 6) *p*-Myceloxybenzoic acid is formed from (3) by the ordinary alkaline breakdown of a substituted benzoylacetic acid leading to the formation of a benzoic and an acetic acid. With ammonia the following reaction must be supposed to occur:

$C_{10}H_{17}.O$—C$_6$H$_4$—$CO.\overset{NH_2}{\underset{R}{\vdots}}H$ → $C_{10}H_{17}.O$—C$_6$H$_4$—$CO.NH_2$.

The nearest analogy in the literature is the production of benzamide from the addition compound of ammonia and benzophenone by the action of water (Houben, 1924). The ammonia produced by the action of caustic soda on mycelianamide arises from the acid amide grouping.

A word or two may now be said on the constitution of the mycelyl radical. Four atoms of hydrogen are readily taken up by the catalytic reduction of mycelianamide in presence of palladium. Hence the mycelyl radical probably contains two double bonds, and the hypothetical mycelyl alcohol ought to be an open-chain diolefinic alcohol, in fact a near relative of geraniol. When mycelianamide was oxidized by ozone the only recognizable product was methylglyoxal (isolated as the *bis*-2:4-dinitrophenylhydrazone), which may mean that the double bonds are conjugated, but may also be interpreted to mean that mycelianamide contains the grouping $CH_3.C.CH_2O.$, which would yield acetol (pyruvyl
‖
alcohol, $CH_3CO.CH_2OH$) by the action of ozone. The latter alcohol would be oxidized to methylglyoxal by the action of dinitrophenylhydrazine. This last explanation implies that mycelianamide is a substituted allyl ether of phenol. The allyl group in such compounds is known to be labile (Claisen, 1912) and the ready formation of mycelene by dilute acid hydrolysis may thus be explained. Mycelene has been examined by Prof. J. L. Simonsen, F.R.S., to whom we desire to express our most sincere thanks, and his observations are included in the experimental section. In brief, mycelene appears to be a monocyclic hydrocarbon containing two double bonds which are not conjugated. One of these double bonds is probably terminal, since formaldehyde is formed in quantity by the action of ozone. It seems very unlikely that mycelene is an isoprene derivative. Owing to the somewhat drastic conditions employed in the production of mycelene from mycelianamide,

the constitution of the former, if known, would provide no certain clues to the constitution of the mycelyl radical in mycelianamide. It is only necessary to recall that the primary alcohol geraniol is converted into the tertiary alcohol linalol merely by heating with water, in order to render probable the supposition that mycelyl alcohol would be an extremely labile substance.

As regards the biochemical origin of mycelianamide, a possible explanation may be that *P. griseofulvum*, which, from our previous researches, is known to be an extraordinarily prolific producer of phenolic substances from glucose, also elaborates the phenolic amino-acid tyrosine far in excess of its requirements. By β-oxidation, amidation, alkylation of the OH group and acylation of the NH_2 group, the excess of tyrosine is converted into an insoluble substance, as long as the medium remains no more alkaline than sodium bicarbonate solution, and is stored in the mycelium. It is significant that mycelianamide is formed when the source of N in the medium is either NO_3^- or NH_4^+, and that the mould produces phenolic substances on both media.

EXPERIMENTAL

History of the culture used

The culture of *Penicillium griseo-fulvum* Dierckx used throughout this work was received in December 1929 from Prof. Ph. Biourge of the University of Louvain, and was numbered *B.* 34. It bears the London School of Hygiene Catalogue No. *P.* 38.

Extraction of mycelianamide

The cultural conditions (as regards modified Czapek-Dox medium with $NaNO_3$ as source of N) and the method of extraction of both griseofulvin and mycelianamide have already been given by Oxford *et al.* (1939). Mycelianamide is best purified by repeated crystallization from ethyl acetate in which it is moderately soluble while hot. The total yield of mycelianamide was more than twice that recorded for griseofulvin in the above memoir, i.e. about 100 g. from 2970 g. of dry mycelium derived from the growth of the mould on about 259 l. of medium containing about 21 kg. of glucose and 650 g. $NaNO_3$. Mycelianamide was also isolated from the mycelium of *P. griseo-fulvum* grown on a medium containing ammonium tartrate as source of nitrogen. The mycelium examined was obtained from our experiments in 1935 (Oxford *et al.* 1935), in which 150 flasks of the following medium were employed: glucose, 50 g.; ammonium tartrate, 5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, 1000 ml. The sterilized flasks, each containing 350 ml. of the above medium, were inoculated with a spore suspension of *P. griseo-fulvum* and incubated at 19–21° for 50 days. The total weight of dried mycelium from three such experiments was 1119 g., yielding 81 g. of crude solid by ether extraction. By crystallization from benzene 51·7 g. of fairly pure mycelianamide m.p. 165° was obtained, containing, however, about 7% of a neutral, amorphous impurity which could not be removed by recrystallization from any solvent. About 20 g. of pure mycelianamide was eventually obtained by extraction with cold very dilute NaOH, immediate acidification of the filtrate, and repeated recrystallization of the dried precipitate. Because of the difficulty of removal of the amorphous impurity it is recommended that the mould be grown on the glucose-$NaNO_3$ medium if it is desired to prepare mycelianamide in quantity.

Analysis and general properties of mycelianamide

Mycelianamide crystallizes from ethyl acetate in colourless, shining leaflets, m.p. 170–172° with vigorous decomposition. (Found: C, 66·09, 66·12, 66·20, 66·15; H, 6·94, 7·12, 7·20, 7·07; N, 7·06, 6·69, 7·33, 7·46%; mol. wt. (cryoscopic in dioxan), 400·5. $C_{22}H_{28}O_5N_2$ requires C, 65·97; H, 7·05; N, 7·00%; mol. wt. 400.) It contains no S, Cl, OCH_3 or NCH_3.

0·1729 g. dissolved in ethanol (10 ml.) and water (3 ml.) required 7·58 ml. 0·1 N-NaOH for neutralization to phenolphthalein; hence equivalent = 228, but the end point was very indefinite and was further complicated by the production of a yellow colour after the addition of the first equivalent of alkali. Some unchanged substance could be obtained by recrystallization of the product obtained by *immediate* acidification of the titrated liquid.

Optical rotation: $[\alpha]_{5461}^{19°} = -217°$; $[\alpha]_{5790}^{19°} = -182°$ (c = 0·8688 in chloroform).

It is sparingly soluble in the cold in ethanol, chloroform, ether, benzene, ethyl acetate and glacial acetic acid; readily soluble in acetone and dioxan.

Mycelianamide is unchanged by boiling water, but is very unstable to cold, dilute NaOH, the solution, which froths readily like a soap solution, soon darkening in colour and acquiring the fragrant lavender-like odour referred to earlier. It is also soluble in Na_2CO_3 solution but insoluble in dilute $NaHCO_3$, $Ba(OH)_2$ or HCl. It forms a series of insoluble *salts* and even the Na salt is partly precipitated by the addition of NaCl to its neutral aqueous solution. The insoluble ferric salt is reddish brown, and the insoluble Cu and Ni salts are green. The yellow insoluble Ag salt becomes black on standing.

Colour reactions. A deep reddish brown colour results when a few drops of very dilute $FeCl_3$ are added to a solution of mycelianamide in ethanol or glacial acetic acid. The colour is discharged by dilute HCl. With 70% H_2SO_4 the colour was first yellow, becoming orange and finally deep brownish red on standing. Mycelianamide does not give the Millon reaction, the murexide reaction, the biuret reaction, or the *p*-dimethylaminobenzaldehyde reaction for indole derivatives. It could not be induced to form a picrate or methiodide, or give any reaction for a $>CO$ group.

Mycelianamide is highly unsaturated and absorbs Br_2 rapidly in chloroform solution. Nothing crystalline resulted from numerous oxidation experiments with $KMnO_4$, H_2O_2, HNO_3 in glacial acetic acid and alkaline I_2. Iodoform was produced in the last instance. Attempted acetylation by acetic anhydride in pyridine solution soon yielded a very dark-coloured liquid from which only amorphous products could be obtained. Methylation by diazomethane yielded a yellow, viscid oil, with an odour of impure acetamide, which was insoluble in both acids and alkalis.

Catalytic reduction of mycelianamide

Mycelianamide (0·832 g.) dissolved in ethyl acetate (100 ml.) was reduced by shaking in an atmosphere of H_2 with a catalyst prepared from palladium chloride (0·1 g.) and norite charcoal (1 g.). Uptake of H_2 was at first rapid (60 ml. in 17 min.) and ceased when 100 ml. had been absorbed in 146 min. (theor. for saturation of two double bonds = 93 ml.). After filtration, the solvent was removed *in vacuo* below 40° to leave a thick brown syrup which became an amorphous solid on treatment with boiling light petroleum, from which nothing crystalline could be obtained. It would be expected that new asymmetric centres might be formed by reduction and the product would then be a complex mixture containing several stereoisomers.

Oxidation of mycelianamide by ozone

Ozonized O_2 was passed through a solution of mycelianamide (0·5 g.) in chloroform (50 ml.) for 6 hr., the temperature being maintained at −10° to 0°. The ozone was completely absorbed throughout and there appeared to be much unchanged substance at the end. The liquid was twice extracted with water and twice with $NaHCO_3$ solution, and the extracts, after acidification with dilute HCl, were added to excess of 2:4-dinitrophenylhydrazine in 2N-HCl. A precipitate slowly formed and was collected after several days. Fine, orange-red needles were obtained by crystallization from aqueous dioxan, m.p. 302° decomp., alone or mixed with the authentic *bis*-2:4-dinitrophenylhydrazone of methylglyoxal. The respective colour reactions with ethanolic KOH were also identical.

Action of hot conc. HCl *on mycelianamide; formation of* NH_4Cl *and the hydrochloride of ω-amino-*p*-hydroxyacetophenone* (I)

Mycelianamide (10 g.) and conc. HCl (1000 ml.; *d* 1·16) were heated together for 15 hr. at 100–105° with frequent shaking. The dark-coloured liquid was then filtered from tar, repeatedly extracted with ether until the extracts were almost colourless, freed from dissolved ether by the passage of a rapid stream of air for several hours, and finally taken to dryness *in vacuo* at 50–60°. The last traces of HCl were removed from the almost black solid residue by repeated shaking with ether. The residue was taken up in a little hot ethanol, and the undissolved part (mostly NH_4Cl) was benzoylated by the Schotten-Baumann method to yield benzamide, m.p. and mixed m.p. 126–128°. Fractional precipitation of the ethanolic extract with ether yielded a crystalline hydrochloride (prisms) and NH_4Cl. The latter, precipitated in a very finely divided condition, came through the filter on suction and was thus removed.

The crude hydrochloride remaining on the filter (1·2 g.) was purified by repeated crystallization from ethanol-ether. It formed almost colourless, compact prisms, m.p. 249–253° decomp., which were readily soluble in water to give a neutral solution which gave a purple colour with $FeCl_3$, an intense yellow colour with bleaching powder solution, a positive Millon reaction but a negative Denigè-Mörner reaction for tyrosine. The compound was the hydrochloride of a primary amine, since an effervescence was immediately observed when very dilute HCl was added to its solution in very dilute $NaNO_2$. The NH_2 was not directly attached to a benzene nucleus since no dyestuff was produced by diazotization and coupling with β-naphthol. Furthermore, the phenolic OH appeared to have a free position *ortho* or *para* to it since an intense orange-red colour was given with diazotized sulphanilic acid. The amine contained no —CHO group since it did not restore the colour to Schiff's reagent.

A *platinichloride* was prepared by addition of 5% platinic chloride solution (10 ml.) to a solution of the hydrochloride (0·15 g.) in water (2 ml.). The mixture was filtered and kept in the cold room when orange needles and prisms separated; m.p. 224° decomp. (Found, on material dried at 15°: C, 25·74; H, 3·31; N, 3·90, 4·02; Pt, 25·89%; loss at 100° *in vacuo*, 4·53%. $(C_8H_9O_2N)_2.H_2PtCl_6.2H_2O$ requires C, 25·65; H, 3·23; N, 3·74; Pt, 26·08; $2H_2O$, 4·81%. Found, on material dried at 100° *in vacuo*: C, 26·94; H, 2·99; Pt, 26·66%. $(C_8H_9O_2N)_2.H_2PtCl_6$ requires C, 26·96; H, 2·83; Pt, 27·39%.)

The amine hydrochloride also yielded a picrate (needles, m.p. 188–190° decomp.) and a benzoate, by the Schotten-Baumann method, which formed long needles from ethanol, m.p. 177–178°. (Found: C, 73·49, 73·30; H, 4·83, 4·77; N, 4·33, 4·21%; mol. wt. (cryoscopic in dioxan), 303. Calc. for $C_{22}H_{17}O_4N$ (dibenzoate of $C_8H_9O_2N$): C, 73·52; H, 4·77; N, 3·90%; mol. wt. 359.)

The free amine is therefore $C_8H_9O_2N$, and the most likely structure which will fit the facts recorded above is $HO.C_6H_4.CO.CH_2.NH_2$.

*ω-Amino-*p*-hydroxyacetophenone* (I) was first made synthetically by Tutin, Caton & Hann (1909) who recorded the following constants: m.p. 190–193° decomp.; hydrochloride, prisms, m.p. 245–252° decomp.; picrate, needles, m.p. 192° decomp.; dibenzoate, needles, m.p. 173–174°. The melting points given for the three derivatives are all close to the values given above for the corresponding derivatives of the amine from mycelianamide. Through the great kindness of Dr T. A. Henry we have been able to make a direct comparison of our hydrochloride with that made by Tutin *et al.* in 1909 at the Wellcome Chemical Research Laboratories. The latter hydrochloride gave a purple colour with $FeCl_3$ and an intense yellow colour with bleaching powder solution; it melted at 246–253° alone or mixed with our hydrochloride, and readily yielded a benzoate of m.p. 176–178°, alone or mixed with the benzoate derived from our hydrochloride. There can thus be no doubt that the amine derived from mycelianamide is *ω*-amino-*p*-hydroxyacetophenone, but, in our opinion, the description of this compound given by Tutin *et al.* (1909) requires amendment in one or two particulars. We have prepared the free amine from their specimen of the hydrochloride, and also from our own, and find that it does not melt at 190–193° as stated, but rather begins to darken at 190° and progressively darkens and softens from 200 to 300°, with production of a sublimate of yellow needles which do not melt at 310°. The amine sublimes readily at 150–170° *in vacuo* to yield a product which behaves exactly as above. (Found on sublimed material from the product obtained by the action of Na_2CO_3 on our amine hydrochloride: C, 63·68; H, 6·07; N, 9·50%. $C_8H_9O_2N$ requires C, 63·54; H, 6·00; N, 9·27%.) Tutin *et al.* (1909) further stated that the platinichloride and aurichloride were both too soluble to admit of isolation. In our experience this statement applies only to the aurichloride.

Hydrolysis with boiling dilute H_2SO_4*; production of* NH_3 (1 *mol.*), CO_2 (1 *mol.*) *and mycelene,* $C_{10}H_{16}$

Mycelianamide (7·85 g.) suspended in a mixture of water (800 ml.) and N-H_2SO_4 (450 ml.) was refluxed for 9·5 hr., in a stream of N_2, until the solid had completely disappeared and had been replaced by a dark-coloured oil floating on a pale-yellow liquid. CO_2 was evolved slowly but continuously throughout and was absorbed in baryta bubblers, the total amount being equivalent to 22·5 ml. of N-$Ba(OH)_2$, i.e. 0·57 mol. of CO_2/mol. of mycelianamide. In a separate experiment, in which the hydrolysis was continued until CO_2 ceased to be evolved, mycelianamide (1·0618 g.) was refluxed with 2N-H_2SO_4 (60 ml.) in a stream of N_2 for 13 hr. CO_2 was evolved equivalent to 53·8 ml. 0·1N-$Ba(OH)_2$. Theor. for 1 mol. CO_2/mol. of mycelianamide, 53·1 ml.

The reaction liquid from the 7·85 g. of mycelianamide was distilled in steam for 5 hr. until no more oil came over.

The acid hydrolysis liquid was treated as follows. A sample was made alkaline and distilled to yield 55% of the N originally present in the mycelianamide, $C_{22}H_{28}O_5N_2$, as a volatile base which was identified as NH_3 by conversion into benzamide, m.p. and mixed m.p. 126–127°. Hence only one of the two N atoms present was accounted for. Part of the remaining N was present in an intractable tar (wt. 0·5 g.) which was formed during the hydrolysis. (Hydrolysis of mycelianamide with 30% (w/w) H_2SO_4 yielded much the same results, save that the amount of tar formed was much greater, and 52% of the N present in the mycelianamide was accounted for as NH_3.) All attempts to isolate crystalline compounds in a state of purity from the hydrolysis liquid failed so that the fate of the second N atom present in mycelianamide remains undetermined. At least two crystalline compounds appeared to be present, viz. clusters of massive plates, m.p. about 150°, and small feathery crystals, m.p. about 100°, both soluble in ether and crystallizing from benzene. Neither substance could be obtained free from adhering oil in quantities sufficient for analysis and characterization. Neither of them was identical with *p*-myceloxybenzoic acid, $C_{17}H_{22}O_3$, m.p. 118–120°, formed in the alkaline hydrolysis (see p. 328).

The steam distillate was treated as follows. A sample was titrated but no volatile acid was detected. The oil was extracted with ether, and the extract dried over anhydrous Na_2SO_4. After removal of the solvent the residual oil was distilled at atmospheric pressure to yield 1·56 g. of distillate, the middle fraction of which (0·68 g.) came over at 177–178° and was almost colourless. By redistillation *in vacuo* it was obtained as a colourless limpid oil with a strong lavender-like odour. (Found: C, 84·26, 84·51; H, 11·21, 11·07; O (by difference), 4·53, 4·42%.) The atomic C : H ratio calculated from the above figures is 1 : 1·57, and since the boiling point is close to that of several terpenes, e.g. *d*-limonene, $C_{10}H_{16}$, boils at 176°, there can be little doubt that the product is essentially a hydrocarbon. The oxygen-containing impurity was not water since the oil submitted to analysis gave an absolutely clear and bright solution in light petroleum.

Several grams of the once-distilled hydrocarbon were prepared and were examined by Prof. J. L. Simonsen, F.R.S., at that time of the University College of North Wales, Bangor, who in a letter dated 24 April 1937, reported as follows.

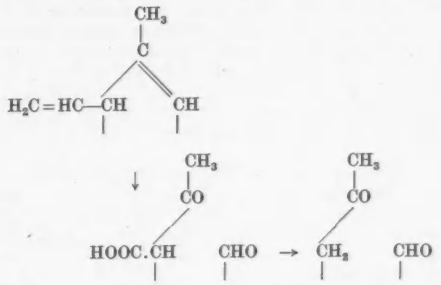
'The hydrocarbon had b.p. 73–75°/20 mm., $d_{25^\circ}^{25^\circ}$ 0·8583, $n_D^{20^\circ}$ 1·4780, $[\alpha]_{5461}=0°$. Molecular refractivity, 45·1; calc. $C_{10}H_{16}$ with two ethylenic linkages 44·6. (Found on sample distilled over sodium: C, 84·6; H, 11·4; after 3 days C, 83·5; H, 11·1; after 4 days C, 83·7; H, 11·0%.) The hydrocarbon is obviously very readily oxidized. It polymerizes to a gum on standing.

The hydrocarbon (3·5 g.) in ethyl acetate (30 ml.) was ozonized at 0°; a considerable quantity of formaldehyde was present in the issuing gases (dimedone test). After removal of the ethyl acetate *in vacuo*, the ozonide was decomposed in water (10 ml.) by heating on the water bath for 1 hr. The oil was extracted with ether, the ethereal extract washed with Na_2CO_3 solution and NaOH, dried over $MgSO_4$ and the solvent removed. A pleasant smelling oil (2 g.) remained. This gave no colour with $FeCl_3$, reduced Fehling's solution and gave a silver mirror (poor). Satisfactory products were not obtained with semicarbazide, phenylsemicarbazide, *p*-nitrophenylhydrazone. The 3:5-dinitrophenylhydrazone separated at once as an amorphous orange-yellow powder. It was very sparingly soluble in ethanol, but crystallized from ethyl acetate-ethanol in what appeared to be rosettes of terracotta prisms, m.p. 170–173° raised to 180–182° by recrystallization. (Found: C, 48·1; H, 4·4; N, 22·1. $C_{20}H_{22}O_8N_8$, i.e. *bis*-dinitrophenylhydrazone of $C_8H_{14}O_2$, requires C, 47·8; H, 4·3; N, 22·3%.)

The acid fractions weighed respectively 0·3 and 0·8 g. Both gave amorphous derivatives with carbonyl reagents, and bromoform with NaOBr. They were combined and oxidized in alkaline solution with this reagent. The resulting acid was a viscid oil. It did not give an anhydride with acetyl chloride; the *p*-bromophenacyl derivative was a viscid gum.

I would suggest that the following conclusions can be drawn tentatively:

(1) The hydrocarbon is cyclic and contains two ethylenic linkages; one of these is exocyclic (formaldehyde) and the other as the group Me.C:CH—. The formation of a keto-aldehyde having the composition $C_8H_{14}O_2$ might result in accordance with the following scheme:



(2) This receives support from the fact that the acid fraction does contain the grouping MeCO— as shown by the NaOBr oxidation with the formation of bromoform. The non-formation of an anhydride suggests that the dibasic acid (if it be such) is an adipic acid.

(3) It seems very unlikely that the hydrocarbon is an isoprene derivative.'

Hydrolysis with moderately strong H_2SO_4; production of acetaldehyde

A mixture of mycelianamide (2 g.) and 30% (w/w) H_2SO_4 (200 ml.) was boiled in a flask connected to a long condenser, the liquid being replaced by distilled water, from a dropping funnel, as fast as it distilled over. After 7 hr. the distillate (900 ml.) was filtered free from oil and redistilled, the first 100 ml. being collected in an ice-cooled receiver. To this was added conc. HCl (10 ml.) and a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl (25 ml.), and the resulting mixture kept overnight at 0°. The precipitated dinitrophenylhydrazone, after two crystallizations from light petroleum, formed shining orange plates, m.p. 161–163°, alone or mixed with the authentic 2:4-dinitrophenylhydrazone of acetaldehyde. A mixed melting point with formaldehyde 2:4-dinitrophenylhydrazone showed a large depression. The yield of acetaldehyde in the above hydrolysis was very small.

Hydrolysis with boiling dilute NaOH, Na_2CO_3 or $NaHCO_3$; formation of NH_3 and p-myceloxybenzoic acid (2; R = OH)

Similar results were obtained with all three alkalis save that the liberation of NH_3 was very slow with $NaHCO_3$ as the hydrolyzing agent. The following is a typical experiment with Na_2CO_3. A solution of mycelianamide (1 g.) in 0·5N-Na_2CO_3 (100 ml.) was very slowly distilled in a current of N_2. After about 3 hr. the distillate no longer turned red litmus blue and the total volatile base which had distilled over was equivalent to 21·8 ml. of 0·1N-HCl, i.e. contained 44% of the N originally present in 1 g. of mycelianamide. The HCl-neutralized distillate, which contained a few drops of mycelene, was taken to dryness *in vacuo* to yield NH_4Cl (fern-like aggregates of needles, m.p. above 300°) which, when benzoylated by the Schotten-Baumann method, yielded benzamide, m.p. and mixed m.p. 126–128°. The brownish red liquid remaining in the distillation flask was acidified with dilute H_2SO_4 and exhaustively extracted with ether to yield a dark-brown syrup, a very small portion of which was soluble in hot light petroleum. Concentration of the petroleum extract yielded colourless slender needles (40 mg.), m.p. 118–120°, after purification by recrystallization from light petroleum. (Found: C, 74·20, 74·39; H, 8·10, 8·10%; N, nil.; mol. wt. (cryoscopic in camphor), 276, 280. $C_{17}H_{22}O_3$ requires C, 74·39; H, 8·09%; mol. wt. 274.)

The product (0·0548 g.) in a mixture of ethanol (6 ml.) and water (4 ml.) required 2·03 ml. of 0·1N-NaOH for neutralization to phenolphthalein; hence equivalent = 270 and the acid is monobasic. The titrated liquid was optically inactive and the unchanged acid was recovered from it by acidification.

The acid $C_{17}H_{22}O_3$ (*p-myceloxybenzoic acid*) is insoluble in water but readily soluble in all organic solvents with the exception of light petroleum. It forms a sparingly soluble Na salt, gives no colour with $FeCl_3$ and only a faint Millon reaction on warming. It could not be induced to form a semicarbazone or 2:4-dinitrophenylhydrazone, and was recovered unchanged after treatment with acetic anhydride in pyridine at room temperature for 2 days.

Hydrolysis of the acid $C_{17}H_{22}O_3$ with production of mycelene and p-hydroxybenzoic acid

The acid (0·6 g.) was treated for 3 hr. with boiling 0·5N-H_2SO_4 (240 ml.) under reflux condenser. One hundred ml. were then distilled off to yield discrete drops of oil in the first runnings, with the characteristic odour of mycelene. The liquid in the distillation flask was cooled, extracted four times with ether, and the extracts dried over anhydrous Na_2SO_4. The yellow crystalline residue (0·3 g.) left after removal of the solvent was crystallized from benzene containing some chloroform to yield colourless flat prisms, m.p. 210–212°, alone or mixed with authentic *p*-hydroxybenzoic acid, m.p. 212°. (Found: C, 61·02, 60·87; H, 4·30, 4·38%. Calc. for $C_7H_6O_3$: C, 60·84; H, 4·38%.)

Action of cold dilute ammonia on mycelianamide; production of p-myceloxybenzamide (2; R = NH_2)

Mycelianamide (50 mg.) was dissolved in N-NH_4OH (6·6 ml.) by gentle shaking, and the solution *immediately* diluted to 200 ml. with distilled water. A crystalline deposit began to appear after standing for 1 day at room temperature and was collected on the fourth day. The filtrate deposited very little more of the product after standing for a further 3 days; total yield, 15 mg. of m.p. about 120°. p-*Myceloxybenzamide* formed colourless silky needles from benzene containing some light petroleum; m.p. 120°, considerably depressed on admixture with *p*-myceloxybenzoic acid, m.p. 118–120°. (Found: C, 74·65, 74·8; H, 8·43, 8·56; N, 5·0, 5·2%. $C_{17}H_{23}O_3N$ requires C, 74·66; H, 8·49; N, 5·1%.) A 1·1% solution in acetone gave absolutely no rotation in a 0·5 dm. tube.

Hydrolysis. The above compound, m.p. 120° (90 mg.), was treated with boiling 20% (w/w) H_2SO_4 (25 ml.) under reflux condenser for 3 hr. Drops of volatile oil were observed in the condenser after a very short time and the fragrant odour was in evidence (formation of mycelene). The reaction liquid was cooled, filtered, extracted once with a little light petroleum and then twice with ether. An almost colourless solid was obtained from the ether extract, which, after treatment with a little boiling benzene and boiling carbon tetrachloride, melted at 210–212°. Mixed with authentic *p*-hydroxybenzoic acid the m.p. was 212–214°. The colour reactions with Millon's reagent and with $FeCl_3$ were identical with those given by *p*-hydroxybenzoic acid.

Bacteriostatic action of mycelianamide

The method of testing used was that described by Oxford (1942) modified to meet the case of a compound insoluble in water but soluble in ammonia. A 1 : 4000 solution of mycelianamide in 0·03N-NH_4OH was made as described above, sterile N-NH_4OH and sterile distilled water being used, and employed *immediately* for the test. Volumes up to 1 ml. of this solution were added aseptically to 4 ml. quantities of sterile 2% glucose broth and the volumes in the tubes made up to 5 ml., if necessary, by the addition of sterile 0·03N-NH_4OH. The controls contained 4 ml. of sterile glucose broth plus 1 ml. of sterile 0·03N-NH_4OH. The inoculum given in all cases was 0·04 ml. of a 1-day culture in glucose broth which had been diluted 1000 times. The tubes were read after 1 day's incubation at 37°. All the controls grew normally. The results are summarized in

Table 1. *Limiting dilutions of mycelianamide for complete inhibition of growth of various bacteria*

1 : 20,000 inhibits	1 : 33,000 inhibits	1 : 50,000 inhibits
Staph. aureus strains* 3093, 3095, 3750 and 3761	*Staph. albus* 3256*	*B. anthracis* 5444*
Strep. viridans 3166*	*Staph. aureus* 4163*	
Strep. pyogenes 2432* (on plain broth)		

* Of the National Collection of Type Cultures.

Table 1, from which it will be seen that *Staphylococcus aureus* and other Gram positive bacteria are completely inhibited by mycelianamide at concentrations of 0·002–0·005%. The compound has no marked action on Gram negative bacteria.

Mycelianamide was equally effective against Gram positive bacteria on plain heart broth containing no added glucose, and almost equally effective if an undiluted culture was used for sowing the tubes. Through the courtesy of Dr A. J. Ewins, F.R.S., we were able to compare the bacteriostatic action of mycelianamide with that of certain aromatic diamidines of comparable molecular complexity, and have found that 4:4′-diamidino-αγ-diphenoxypropane ($C_{17}H_{20}O_2N_4$; mol. wt. =312) is almost identically potent when tested in dilute aqueous solution, in that 1 part in 40,000 would inhibit the growth of *Staph. aureus* No. 3095, and 1 part in 20,000 would inhibit the growth of *Staph. aureus* No. 3750 for 1 day.

SUMMARY

1. A hitherto undescribed, colourless, crystalline, metabolic product, *mycelianamide*, $C_{22}H_{28}O_5N_2$, m.p. 170–172° decomp., has been isolated from the mycelium of *Penicillium griseo-fulvum* Dierckx.

2. Acid hydrolysis of mycelianamide under different conditions gives a new hydrocarbon, *mycelene*, $C_{10}H_{16}$, ω-amino-*p*-hydroxyacetophenone, acetaldehyde, ammonia and carbon dioxide.

3. Alkaline hydrolysis gives ammonia and a monobasic acid, $C_{17}H_{22}O_3$, which on acid hydrolysis affords mycelene and *p*-hydroxybenzoic acid.

4. Consideration of these and other reactions leads to the tentative conclusion that mycelianamide may be the *amide* of O-*mycelyl*-N-*pyruvyl*-β-*keto-tyrosine*.

5. Mycelianamide completely inhibits the growth of a number of Gram positive bacteria *in vitro* at concentrations of 1 : 20,000 to 1 : 50,000, but has no marked action on Gram negative bacteria.

We desire to thank Prof. J. L. Simonsen, F.R.S., for his examination of the hydrocarbon, mycelene; Dr A. J. Ewins, F.R.S., for a gift of aromatic diamidines; and Dr T. A. Henry for a gift of ω-amino-*p*-hydroxyacetophenone.

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Biochemistry of the Wood-rotting Fungi

5. THE PRODUCTION OF D-THREITOL (*l*-ERYTHRITOL) BY *ARMILLARIA MELLEA* (VAHL) QUÉLET

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The tetrahydric alcohol until recently known as erythritol is capable of existing in three stereoisomeric forms (if we exclude external compensation), the optical relationships being the same as for tartaric acid. The optically inactive internally compensated form, known as *meso*erythritol or *i*-erythritol has frequently been observed in nature. It is found in the algae *Protococcus vulgaris* (Lamy, 1852) and *Trentepohlia Jolithus* (Bamberger & Landsiedl, 1900). It also occurs in lichens of the genus *Roccella* (Stenhouse, 1848; Hesse, 1904) usually in the form of erythrin from which it can be obtained in the free state by hydrolysis. It has also been isolated from various fungi. Zellner (1910) obtained it from spores of *Ustilago maydis*, Oxford & Raistrick (1935) from the mycelium of *Penicillium brevi-*

compactum Dierckx and of *P. cyclopium* Westling, and Stodola (1946) from *Aspergillus terreus* Thom.

There is, however, no record in the literature of the natural occurrence of the optically active forms of erythritol, although they have been prepared by synthetic methods by Maquenne (1900) and others. This gap has now been partially filled by the isolation from a natural source of the form of erythritol which is dextrorotatory in water and laevorotatory in ethanol and which in accordance with the modern nomenclature is described as D-threitol. It was obtained in a yield of about 13% on dry weight from the mycelial tissue of a freshly isolated laboratory culture of *Armillaria mellea* (Vahl) Quélet, one of the higher fungi. When subcultured in the laboratory under our conditions, the fungus rapidly lost its power of producing D-threitol. The species is widely distributed and has been reported as a cause of damage to a variety of plants ranging from forest trees to shrubs and herbaceous plants. It is very common on tree stumps, but may become parasitic, attacking the base of living trees.

The new product is apparently identical with the substance synthesized by Maquenne from natural xylose and described under the name of '*l*-erythritol'. The name was later changed to '*d*-erythritol' in accordance with the Rosanoff convention for sugars, but recently it has been thought advisable to change the name to D-threitol to relate it more closely to the tetrose threose.

EXPERIMENTAL

Culture. The culture was obtained by transplantation on to malt agar of a fragment of tissue from the fruiting body of *Armillaria mellea* found growing in a large clump at the foot of a horse-chestnut tree at Pinner, Middlesex. The inoculum was taken under sterile conditions from a newly exposed surface obtained by breaking the pileus.

Medium and conditions of culture. Two culture solutions were prepared from a basal medium of the following composition: glucose 30 g.; KH_2PO_4 2·5 g.; $MgSO_4.7H_2O$ 1·0 g.; $FeSO_4.7H_2O$ 0·01 g.; $ZnSO_4.7H_2O$ 0·01 g.; $MnSO_4.4H_2O$ 0·005 g.; $CuSO_4.5H_2O$ 0·005 g.; $Na_2B_4O_7.10H_2O$ 0·005 g.; NH_4Cl 2·5 g.; water 1000 ml. For culture medium *A* an addition of 10 g. of corn-steep powder, and for medium *B* an addition of 0·1 g. of Marmite was made.

The medium was distributed in conical flasks of 1 l. capacity, 350 ml. to each flask. It was sterilized by autoclaving at 110° and inoculated with fragments of tissue from slopes on malt agar containing an addition of 1% of corn-steep solids. In culture on liquid media of depth about 1 in. the fungus forms a mass of tangled rhizomorphs, the lower portion of the mass eventually coming to rest on the bottom of the vessel and the upper portion appearing above the surface of the liquid.

Growth and harvesting of fungus. The rate of growth and utilization of sugar is much more rapid on medium *A* (corn steep) than on medium *B* (Marmite). The contents of single flasks were harvested after varying intervals of time. The culture solution was filtered from the total mycelium obtained from the six single flasks (Table 1). The mycelium was dried *in vacuo* (dry weight 20·00 g.), powdered and subjected to continuous extraction. After a preliminary extraction with light petroleum to remove lipid material the solid was subjected to exhaustive extraction with dry ether (16 working days). Even after this length of time, matter was still being extracted, but the daily yield had fallen to less than 0·1 g. of extractive. During the extraction a crystalline substance separated on the walls of the extraction flask and was scraped off daily before renewing the extraction. Evaporation of the ether gave a little more of this crystalline material (0·12 g.) and 0·25 g. of a non-crystalline oil. The extraction was continued with ethyl acetate, but only a further 0·05 g. of the crystalline solid was obtained; the total weight of crystals extracted was 2·62 g. or 13% of the dry weight of mycelium.

Table 1. *Rate of growth and utilization of glucose by* Armillaria mellea *on synthetic media with added corn-steep solids* (*A*) or *Marmite* (*B*)

	Dry mycelium (g./flask)		Residual glucose by polarimeter (%)	
Medium	*A*	*B*	*A*	*B*
Period of growth (days)				
11	3·02	—	2·05	—
24	4·82	1·32	0·09	2·78
35	6·82	1·79	0·00	2·28
45	—	2·23	—	2·11

Identification of the crystalline solid as D-*threitol.* The crystalline solid was recrystallized from absolute ethanol and was thus obtained in the form of long colourless silky needles with a sweet taste. The m.p. was 90–91° (corr.), not changed by further recrystallization. The product dissolved readily in water to give a neutral solution, and gave a strong reaction for primary or secondary hydroxyl groups in the test of Fearon & Mitchell (1932). (Found: C, 39·4, 39·5; H, 8·3, 8·3. Calc. for $C_4H_{10}O_4$: C, 39·4; H, 8·3%.) Tests for N, S, and halogen were negative. The substance was found to be optically active; the following rotations were observed. In 90% ethanol, $[\alpha]_{5780}^{20°} = -13·3°$, $[\alpha]_{5461}^{20°} = -14·5°$ ($c = 5\%$). In water, $[\alpha]_{5780}^{20°} = +4·7°$, $[\alpha]_{5461}^{20°} = +5·5°$ ($c = 5\%$).

Dibenzylidene derivative. When the substance (0·1 g.) was treated with conc. HCl (2 ml.) and benzaldehyde (0·2 ml.) a colourless crystalline solid, insoluble in water, was obtained. This was collected, washed, dried and recrystallized from ethanol, then from benzene, and was thus obtained in the form of needles of m.p. 215–222° (corr.). (Found: C, 72·2; H, 6·2. Calc. for $C_{18}H_{18}O_4$: C, 72·5; H, 6·1%.)

The properties of the fungal product are thus in general agreement with those described by Maquenne (1900) for '*l*-erythritol' now known as D-threitol. Complete identity of the product with D-threitol was established by the synthesis of the latter substance.

Preparation of D-*threitol.* The method chosen was that of Hockett (1935) who prepared dibenzylidene-D-threitol via D-threose from D-xylose. The dibenzylidene compound was hydrolyzed in aqueous H_2SO_4 to yield D-threitol.

Tetra-acetyl-D-xylononitrile. Early experiments gave very low yields even when the reactants were vigorously dried. The following reacetylation procedure increased the yield from about 20 to 52% of theoretical (Hockett (1935) claims 48%).

After the acetic anhydride had all been added the mixture was held at 70–75° for 15 min. and evaporated at this temperature under reduced pressure. When most of the solvent was removed a volume of acetic anhydride equal to half that originally employed was added. After 15 min. at 70–75° the solvent was evaporated and the procedure repeated with fresh acetic anhydride. After the final evaporation water was added and the mixture was boiled for 0·5 min. to decompose acetic anhydride. On cooling, the product solidified. It was collected, washed, dried and recrystallized from ethanol. It had m.p. 82–83° and was coloured slightly brown, but was sufficiently pure for the next stage.

D-Threose diacetamide. As the purification method of Hockett gave only a low yield it was decided to dispense with purification at this stage. The crude product, after evaporation of NH_3, partly crystallized on standing.

Dibenzylidene-D-threitol. The crude D-threose diacetamide was hydrolyzed in 0·1 N-H_2SO_4 at 100° for 6 hr. the solution being maintained acid to congo red by addition of 2 N-H_2SO_4 as required. The deep red solution was reduced with Na amalgam. Complete reduction (as indicated by Fehling's test) could not be effected, but when 80–90% reduction was attained the solution was adjusted to pH 6 and evaporated to small bulk. Ethanol was added and the mixture was cooled and filtered. The filtrate was evaporated to dryness, and the residue was taken up in conc. HCl (10 vol. based on the theoretical content of D-threitol) and shaken with benzaldehyde (2 vol. on same basis); the dibenzylidene derivative separated at once. The mixture was poured into ice and ether, warmed to 0° and the product collected, washed and dried. Yields were very variable and never greater than 23% of theoretical, based on nitrile, the best yields being obtained with small scale preparations using up to 12 g. of the nitrile. Recrystallized from benzene the product had m.p. 217–222° (corr.).

D-Threitol. The dibenzylidene compound (3·5 g.) was added to 2 N-H_2SO_4 (100 ml.), the mixture boiled until solution was complete and the benzaldehyde distilled off in steam. A slight excess of $CaCO_3$ was added, and the mixture was filtered. The filtrate was adjusted to pH 6 with H_2SO_4 and evaporated to dryness. The D-threitol was extracted from the solid residue by boiling with ethanol. The ethanol was removed and the residue crystallized from *n*-butanol. Yield 0·94 g. (66%), m.p. 90–91° (corr.). A further 10% was obtained from the mother liquors. The melting points and specific rotations of the natural and synthetic D-threitol and of their benzylidene derivatives are shown in Table 2.

Table 2. *Comparison of natural and synthetic* D-*threitol*

	D-Threitol							Dibenzylidene-D-threitol			
		Optical rotation							Optical rotation in chloroform ($c=1\%$)		
		In water ($c=5\%$)			In 90% ethanol ($c=5\%$)						
	Melting point	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$	Melting point	$[\alpha]_{5461}^{20°}$	$[\alpha]_{5780}^{20°}$	$[\alpha]_{D}^{20°}$
Natural	90–91° (corr.)	+5·5	+4·7	—	−14·5	−13·3	—	215–222° (corr.)	−97	−84	—
Synthetic (our observation)	90–91° (corr.)	+5·5	+4·5	—	−14·3	−13·0	—	217–222° (corr.)	−96	−87	—
Mixture of above	90–91° (corr.)	—	—	—	—	—	—	217–220° (corr.)	—	—	—
Synthetic (Maquenne, 1900)	88°	—	—	+4·33 ($c=6\%$)	—	—	−10·50	231°	—	—	—
Synthetic (Hockett, 1935)	—	—	—	—	—	—	—	218–222°	—	—	−77·9
	—	—	—	—	—	—	—	220–222° (corr.)	—	—	−78·2

Rotation figures liable to an error of ±5%.

The figures for the melting point of the dibenzylidene compound of threitol quoted in the literature show a considerable range: 231° (Maquenne, 1900), 204–205° (Ruff, 1901), 218–222°, 220–222° (Hockett, 1935). Since the preparation of the compound introduces two new asymmetric carbon atoms into the molecule it is probable that the substances as obtained after recrystallization are mixtures of different stereoisomers. Melting points of dibenzylidene derivatives of other polyhydric alcohols quoted in the literature also show wide variations. The only reference found to this possibility, however, is in a paper by Radulescu & Tanasescu (1924) where two forms of *bis-o*-nitrobenzylidene-erythritol and two of *bis-m*-nitrobenzylidene-erythritol are described.

SUMMARY

A freshly isolated culture of the wood-rotting fungus *Armillaria mellea* (Vahl) Quélet, grown on a liquid culture medium, gave rise to D-threitol in the mycelium, the yield being about 13% of dry weight.

We have to thank Sir Norman Haworth, F.R.S., for valuable advice on the nomenclature of the polyhydric alcohols. One of us (C.E.S.) is indebted to the Therapeutic Research Corporation of Great Britain Ltd. for a grant which has enabled him to take part in this work.

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An Alkali-producing Mechanism in Macerated Leaves

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While investigating the enzymic demethylation of pectin in macerated leaves, a phenomenon not previously described was observed in some members of the Cucurbitaceae (Holden, 1945). When the fibre residue, obtained by squeezing out the sap from minced leaves, was washed with distilled water, it was found that successive washes had higher pH values until a maximum of pH 9·4–9·6 was reached, and this value was maintained on continued washing. The present paper concerns further investigations of this spontaneous rise of pH. The phenomenon would appear to be of importance in the extraction of normal and virus proteins from green leaves since the infectivity of a number of plant viruses is decreased by pH values greater than 9.

MATERIAL AND METHODS

The plants examined were obtained from various sources. All the Cucurbitaceae (except marrow, cucumber and bryony) were from the University Botanic Garden, Cambridge. *Nicotiana tabacum* and *N. glutinosa* were glasshouse grown and the rest of the plants were from gardens or were found growing wild in the neighbourhood of Rothamsted.

The leaves were minced with a domestic meat mincer and the sap squeezed out by hand through madapollam. The fibre was washed by suspending in distilled water (about 5 ml./g. fibre wet weight) and again squeezing out. Measurements of pH were made with a glass electrode.

Calcium was determined on ashed material. Ashing was done in porcelain crucibles over a Bunsen burner at *c.* 500° on weighed portions of fibre or measured volumes of sap that had been dried at 100°. The dried material was moistened with a few drops of HNO_3 to assist ashing. After weighing, the ash was dissolved in N-HCl, filtered, and calcium precipitated as oxalate; the latter was dissolved in H_2SO_4 and titrated with $KMnO_4$ (0·05 N or 0·01 N depending on the amount of oxalate).

Carbonate determinations were made by the method of Hutchinson & MacLennan as described by Piper (1942) except that $Ba(OH)_2$ was used instead of NaOH to absorb the CO_2 liberated.

Phosphorus was determined colorimetrically by a modification of the method of Kuttner & Lichtenstein (1932). Values for inorganic phosphorus were obtained by developing the colour in samples without previous incineration.

EXPERIMENTAL AND RESULTS

Plants showing alkaline drift

Plants of different families, including ten genera of the Cucurbitaceae, were examined and are listed in Tables 1 and 2. As the pH of the expressed sap from the leaves in which the pH rise was first observed was neutral or slightly alkaline, in contrast to the somewhat acid saps found in most plants, other plants known to have more alkaline sap were examined. Haas (1920) had recorded a pH as high as 8·5 for the expressed sap of sweet clover leaves. These were not available, but the sap of common yellow melilot (*Melilotus altissima*), which is closely related, had a pH of 7·8. On washing the fibre, however, the pH fell to 6·3. The pH of sunflower-leaf sap was given by Gustafson (1924) as between 6·3 and 6·9. This was confirmed and both sunflower (*Helianthus annuus*) and Jerusalem artichoke (*H. tuberosus*) were found to show the pH rise, though other members of the Compositae that were examined did not. All other members of the Cucurbitaceae as well as comfrey (*Symphytum officinale*) of the Boraginaceae and stinging nettle (*Urtica dioica*) of the Urticaceae showed the phenomenon. Although the pH tended to be higher in sap from leaves which showed the drift than from those which did not, this was not always so as can be seen from Tables 1 and 2. Young leaves either did not show the pH rise at all or only to a much less marked extent.

Fig leaves (*Ficus carica*) were anomalous in that the pH rose to 8 but no higher; these cannot therefore be included in either group.

Table 1. *Calcium distribution in plants not showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Solanaceae	Tobacco	*Nicotiana tabacum*	6·05	8·7	1·5	1·6	52·5
		Nicotiana glutinosa	5·28	8·9	8·2	1·8	16·8
	Tomato	*Lycopersicum esculentum*	6·20	10·6	3·7	1·9	35·0
Leguminosae	Melilot	*Melilotus altissima*	7·79	20·2	1·9	2·3	61·2
Cruciferae	Broccoli	*Brassica oleracea*	5·80	17·3	3·9	1·7	44·3
Labiatae	Dead-nettle	*Lamium purpureum*	6·11	16·3	1·4	0·9	48·7
Rosaceae	Strawberry	*Fragaria vesca*	—	32·9	0·5	1·3	82·9
Compositae	Marigold	*Calendula officinalis*	5·93	7·3	0·9	0·9	19·6
	Groundsel	*Senecio vulgaris*	5·86	8·0	1·4	0·8	38·6
	Chrysanthemum	*Chrys. hortorum*	5·80	19·6	2·5	1·4	47·3
Labiatae	Peppermint	*Mentha piperata*	6·30	14·5	5·3	1·0	44·5
Caprifoliaceae	Elder	*Sambucus nigra*	5·85	19·7	0·9	2·3	72·0

Table 2. *Calcium distribution in plants showing alkaline drift*

Family	Common name	Latin name	pH of sap	Dry wt. as % wet wt.	Ca as % dry wt. of sap	Ca as % dry wt. of fibre	% total Ca in fibre
Cucurbitaceae	Marrow	*Cucurbita ovifera*	7·00	16·1	1·7	10·2	90·3
	Cucumber	*Cucumis sativus*	7·43	11·7	4·7	11·3	85·2
		Sicyos angulata	6·29	7·6	4·2	5·3	50·0
	Calabash	*Lagenaria leucantha* v. *longissima*	6·15	11·8	6·3	4·2	38·0
		Momordica charantica	6·61	24·1	0·7	7·5	90·5
	Squirting cucumber	*Ecballium elaterium*	6·80	11·1	2·5	6·9	66·1
	Loofah	*Luffa cylindrica*	5·50	19·7	1·8	6·1	77·8
	Bryony	*Bryonia dioica*	6·98	11·1	1·1	5·9	91·3
		Thladiantha dubia	—	13·9	—	6·1	—
		Cyclanthera explodens	—	12·0	—	5·9	—
Compositae	Sunflower	*Helianthus annuus*	6·60	*c.* 15·0	2·3	7·3	85·3
	Jerusalem artichoke	*Helianthus tuberosus*	6·11	20·3	1·5	6·2	85·5
Urticaceae	Nettle	*Urtica dioica*	—	21·8	3·0	6·5	90·0
Boraginaceae	Comfrey	*Symphytum officinale*	7·00	13·2	0·5	3·6	96·0

The leaves of all species showing the alkaline pH drift have a characteristic dry and rough feel due to the covering of large hairs which are a conspicuous feature of all of them. A microscopical examination of sections stained in ethanolic purpurin to distinguish calcified areas (Baecker, 1930) showed that both uni- and multicellular hairs and the walls of cells in the region of the hairs were calcified. In addition cystoliths occurred beneath the upper epidermis in *Urtica dioica*.

General observations on the pH *drift*

Water volume. When the maximum pH value is reached, the volume of water used for washing does not influence the pH to any great extent. In one experiment, a wash with 10 ml. water/g. wet weight nettle fibre had a pH of 9·5 while a wash with 200 ml./g. had a pH of 9·6. No volatile alkali was found in alkaline washes, but an appreciable amount of calcium (0·15–0·35 mg./ml.), traces of phosphorus (2–4 μg.) and magnesium were present.

Milling. When minced fibre is finely ground in a triple-roller mill (Bawden & Pirie, 1944) the pH is raised even without previous washing. In some species, a pH of 9 or higher is not reached by washing minced fibre but only after grinding. The pH of fibre of leaves which do not show the high pH is raised by about 0·2 unit on milling.

Heating. The pH rise is not prevented by boiling a suspension of fibre; it may actually be speeded up and the maximum pH may be higher. Whole leaves dipped into boiling water gave an extract with a pH between 8 and 9. The action appears therefore not to be enzymic.

Effect of KCl *and* $CaCl_2$. Minced fibre washed with N-KCl solution instead of water gave the alkaline pH drift, but the soaking of fibre in 0·2 M-$CaCl_2$ solution tended to prevent the pH rise.

Alkali content of fibre. It was found that addition of dilute acid to fibre caused effervescence and evolution of CO_2. When 0·2 N-HCl was added to a minced fibre suspension to lower the pH to 3·5, the pH rose after a short time to between 7 and 8 and it

only remained stable at 3·5 after several additions of acid. The pH did not rise above 7 between additions of acid unless the solution was squeezed out from the fibre and the fibre resuspended in water, when the pH rose to above 9. Milled fibre took up a large amount of acid in one addition and no pH rise occurred.

Calcium was found in the acid extract and quantitative determinations showed that it was present in an amount sufficient to account for most of the acid added. The calcium content of the sap washes and of the fibre was determined in the various species and the results expressed on a dry matter basis. The results given in Tables 1 and 2 show that the 'alkaline drift' plants have a much higher calcium content than those of the other group. The calcium content of the sap as a percentage of dry matter is similar in both groups, but the fibre calcium is considerably higher in plants giving the pH rise; the proportion of the total calcium which is insoluble is thus greatly increased.

The calcium content of the fibre of young leaves of 'alkaline drift' plants is lower than that of fully grown leaves. Young bryony leaves (picked in June) had a fibre calcium of only 2·2 % dry matter whereas the larger older leaves from the same plants had a value of 6·2 % dry matter.

The carbonate content of milled marrow fibre was found to account for 80 % of the total calcium content. The greater part of the calcium is thus present as $CaCO_3$.

Phosphorus content of fibre. Phosphorus determinations were made on fibres of a number of species. The P content of 'alkaline drift' fibres was considerably higher than that of fibres which do not show the drift. Table 3 gives a comparison of the P distribution in fibre of nettle and tobacco, as examples of both types of fibre.

Portions of fibre (0·5 g.) were extracted with 10 ml. 0·2 N-HCl, centrifuged after 2 hr. and a further 5 ml. acid added. After the second portion of acid had been decanted, 10 ml. ethanol-ether (7 : 3) were added to remove lipid P. After a further 2 hr., a second extraction with a 5 ml. portion of ethanol-ether followed and the fibre was then dried. (HCl (0·2 N) was used for acid extraction in the earlier experiments, but later cold 10 % trichloroacetic acid was used as recommended by Schneider (1945) for fractionation of P in animal tissue.) Total and inorganic P were determined on the acid extract, total P on the ethanol-ether extract and on the fibre residue.

The amount of inorganic P in nettle fibre is remarkably high but even higher values of up to 1·2 % of the dry matter have been found. The highest value for tobacco fibre was less than 0·4 % and these plants had been given a large amount of phosphate fertilizer. The P content of the fibre of young leaves is higher than that of old even in 'alkaline drift' plants, but the amount of inorganic P is less. Table 4 compares the P distribution in the fibre of old and young nettle leaves picked in June.

Thoday & Evans (1932) stated that the phosphate in *Helianthus tuberosus* was localized in the multi-

Table 3. *Comparison of fractionation of fibre* P *in tobacco and nettle*

Fraction	Tobacco			Nettle		
	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·240	100	—	0·720	100	—
Extracted by 0·2 N-HCl	0·103	43	100	0·634	88	100
Inorganic P in acid extract	0·072	17	70	0·570	79	90
Ethanol-ether (7 : 3) extractable	0·024	10	—	0·043	6	—
Residue	0·113	47	—	0·043	6	—

Table 4. *Comparison of* P *fractionation in young and old bryony leaves*

Fraction	Young leaves			Old leaves		
	P as % total dry wt.	Total P (%)	Acid-extractable P (%)	P as % total dry wt.	Total P (%)	Acid-extractable P (%)
Total	0·650	100	—	0·420	100	—
Extracted by 10 % trichloroacetic acid	0·514	79	100	0·294	70	100
Inorganic P	0·108	16·6	21	0·265	63	90
Ethanol-ether (7 : 3) extractable	0·059	9	—	0·063	15	—
Residue	0·078	12	—	0·063	15	—

cellular epidermal hairs and in *H. annuus* in the basal cells of the hairs and the cortical cells below. The method they used involved treatment of sections with a solution of ammonium molybdate in HNO_3 which produced a yellow crystalline precipitate with the phosphate in the tissues; this was then reduced to a bluish-black oxide of molybdenum with phenylhydrazine hydrochloride. A microscopical examination of minced fibre of 'alkaline drift' plants showed that there were large numbers of undamaged epidermal hairs. When marrow leaf fibre was treated by the above method it gave a large quantity of yellow crystals which were concentrated to some extent in and around the hairs, but owing to the acidity of the reagent, which must be maintained, much phosphate was extracted from the fibre into solution. It seems probable that much of the calcium and phosphate are localized in the same cells and that in addition to calcium carbonate some of the calcium is present as a calcium phosphate.

Conditions under which a pH *rise takes place with 'non-drifter' fibre*

A pH rise could be obtained with 'non-drifter' fibre by adding $CaCO_3$ and phosphate and, when the necessary conditions had been established, the investigation was continued to find which constituent

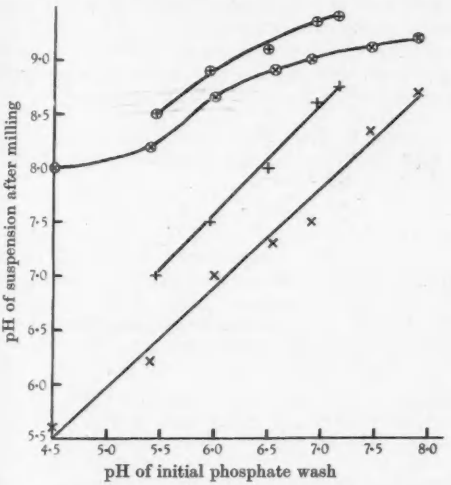

Fig. 1. The effect of milling fibre 'phosphated' at different pH values. ×—× nightshade fibre; ⊗—⊗ nightshade fibre + $CaCO_3$; +—+ tomato fibre; ⊕—⊕ tomato fibre + $CaCO_3$.

of fibre, in addition to $CaCO_3$ and phosphate, could be responsible. When tobacco leaf fibre was milled with $CaCO_3$ the pH did not rise above that of the $CaCO_3$ itself (i.e. pH 8). When, however, it was neutralized by soaking in 0·2 M-Na_2HPO_4 adjusted to pH 7·9, washed with water and then milled with $CaCO_3$, a pH of 9·3 was obtained. Unless the fibre was thoroughly washed after 'phosphating', the pH rise did not occur, due undoubtedly to the buffering action of the phosphate. 'Phosphated' washed fibre had a P content about twice the original value. Fibre which had been soaked in phosphate solution and milled without $CaCO_3$ showed a rise but the pH was not as high as with the $CaCO_3$. The actual pH reached on milling, with or without $CaCO_3$, was found to depend on the pH of the phosphate solution in which the fibre had previously been soaked. The results of an experiment using nightshade (*Solanum dulcamara*) and tomato fibre are shown in Fig. 1.

A similar effect could be obtained by replacing sodium phosphate as a soaking fluid by sodium citrate or potassium oxalate but not by sodium acetate, sodium sulphate or sodium chloride. The pH rise was also obtained with acid-extracted nettle fibre under the same treatment. Table 5 gives the results of an experiment using minced nettle fibre.

Table 5. *Reconstitution of* pH *rise in acid-extracted nettle fibre*

(15 g. minced nettle fibre soaked in 100 ml. N-HCl overnight, squeezed out and washed twice with 100 ml. water. pH of fibre suspension 2·7.)

Treatment	pH
Fibre (3 g.) milled	5·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	7·4
Fibre (6 g. in 40 ml. 0·2 M-Na_2HPO_4) squeezed out and washed	7·9
Fibre (3 g.) milled	8·4
Fibre (3 g.) milled with 1 g. $CaCO_3$	9·7

The constituent of the leaf responsible is not protein since incubation with a commercial trypsin preparation removes a large proportion of the nitrogenous constituents from tobacco fibre but does not prevent the pH rise. Cellulose also does not appear to be involved since experiments with filter paper (treated with alkali to render it suitable for milling, and then well washed) do not give the effect. When, however, pectic acid was removed from fibre it was not possible to obtain a pH rise.

Tobacco fibre (10 g., dry wt. 2·6 g.), suspended in water, was incubated at 40° and pH 5 for 48 hr. with 1 mg. of a preparation of polygalacturonase (purified from a commercial pectinase of fungal origin) to convert pectic to galacturonic acid (Jansen & MacDonnell, 1945). The fibre lost 450 mg. of carbohydrate and, when soaked in neutral phosphate solution, washed and milled with $CaCO_3$, did not show a rise above pH 8. The same effect was brought about by incubation with the crop juice of *Helix aspersa* and was due presumably to its polygalacturonase action. As the enzymic removal of pectic acid from fibre thus abolished the effect, experiments were done with pectic

acid. This was prepared by de-esterification of citrus pectin (British Drug Houses Ltd., 100 grade) enzymically with tobacco pectase and also with 0·1 N-NaOH. The pectic acid (0·5 g.) was soaked in 25 ml. 0·2 M-Na_2HPO_4 solution for 2 hr. during which a small amount dissolved. The undissolved portion was centrifuged down and then washed three times with 50 ml. water. $CaCO_3$ (0·5 g.) was then added which caused a pH rise to 9, and when the paste of pectic acid and $CaCO_3$ was milled the pH obtained was 9·8. A similar experiment, in which 0·5 g. pectic acid and 0·5 g. $CaCO_3$ were soaked in phosphate solution and the solid material then washed, showed a pH rise to 9·2 by the fourth wash and to 9·9 when milled.

Pectic acid thus behaves in the same way as whole fibre of 'non-drifter' leaves when soaked in phosphate solution, washed and milled with $CaCO_3$, and the phenomenon is similar to that originally observed with the fibre of leaves rich in calcium carbonate and in phosphate.

DISCUSSION

Wilkins (1917) reported very high values for the calcium content of various members of the Cucurbitaceae, but made no suggestion as to its mode of combination. Smith (1944) stated that 70 % of the calcium of squash leaves was immobilized in cell walls or intracellular substances and was readily extracted with dilute acid. It was also observed that the cell wall residue after various extraction treatments when suspended in water had a pH of 9·0.

Although the presence of calcified areas in plant tissue has been known for a considerable time, it has not been observed that a large proportion of the total calcium and as much as 10 % of the dry weight of the leaves of some species is calcium carbonate. The amount of insoluble inorganic phosphate in 'alkaline drift' plants, presumably as calcium phosphate, is also noteworthy.

Two species which show the pH rise, nettle and sunflower, were those investigated by Spoehr and his coworkers from 1923 to 1940 (see Spoehr, Smith, Strain & Milner, 1940; and Smith, 1940) on account of their ability to absorb carbon dioxide when killed by drying or freezing. Smith (1940) concluded that the absorptive agents were calcium and magnesium carbonates, and possibly phosphates.

SUMMARY

1. A pH rise to *c.* 9·5 on washing the fibre of macerated leaves was observed in all species of the Cucurbitaceae examined and some members of the Urticaceae, Boraginaceae and Compositae.

2. The rise is not prevented by heating and is speeded by fine grinding.

3. 'Alkaline drift' plants have a high calcium content, a large proportion of which is insoluble and occurs as calcium carbonate. The calcium content of the fibre may be as high as 11 % of the dry matter and the inorganic phosphorus content is also high, up to 1 % of the dry matter. The calcium and phosphorus appear to be localized in and near the epidermal hairs.

4. Young leaves which have much lower contents of calcium and inorganic phosphorus do not show much pH rise.

5. A pH rise to >9 can be produced with 'non-drifter' and acid-extracted 'drifter' fibre and with pectic acid by soaking in neutral phosphate solution, washing and milling with calcium carbonate. After incubation of 'non-drifter' fibre with polygalacturonase the pH rise cannot be obtained.

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Effects of Organic Arsenicals on Enzyme Systems

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Investigations have been carried out by us in the Biochemical Laboratory of the Cardiff City Mental Hospital during 1938, 1939 and early 1940 on the effects of organic trivalent and pentavalent arsenic compounds on proteins and enzymes. The work was primarily undertaken to elucidate the biochemical basis of the trypanocidal and anti-spirochaetal action of a variety of organic arsenic compounds used in the treatment of syphilis. Investigations by Hawking, Hennelly & Quastel (1937) had already shown that administration of the pentavalent arsenical tryparsamide into the body led to the appearance of trivalent arsenic compounds, of high trypanocidal activity, in the spinal fluid. On the other hand, administration of organic trivalent arsenic compounds did not lead to the excretion of active trivalent arsenic compounds in the spinal fluid. Work was therefore undertaken to discover the manner of combination of organic trivalent and pentavalent arsenic compounds with tissue proteins and enzyme systems. It was inevitable that our work led us into problems connected with the mechanism of action of certain war gases (e.g. lewisite) and with the question of reversibility of enzyme systems inactivated by arsenic compounds. Whilst we, ourselves, did not work with actual war gases, our results bore on the problems involved and for this reason publication of our results was not allowed during the war. A report, however, was submitted to the Medical Research Council in November 1940, and an extract containing the main conclusions was published in *Nature* (Gordon & Quastel, 1947) by permission of the Secretary of the Medical Research Council.

The present paper contains the results of investigations made by us on the effects of organic arsenic compounds on enzyme systems during 1938, 1939 and early 1940; they are the results on which the conclusions, already published by Gordon & Quastel (1947), are based.

Much attention has been devoted by workers in chemotherapy to the elucidation of the mechanism of the trypanocidal and anti-spirochaetal activity of arsenic compounds. It is a well established fact, first noted by Ehrlich, that while pentavalent arsenic components are relatively non-toxic, *in vitro*, to protozoa, trivalent arsenic compounds such as substituted phenylarsenoxides and arsenobenzenes are extremely toxic. Moreover, there is good reason to believe that, in the case of the arsenobenzenes, the toxic action is not exerted to any great extent by these compounds as such, but is probably due to the presence, in their aqueous solutions, of highly toxic arsenoxides, rapidly formed by atmospheric oxidation of the —As=As— group. Eagle (1939*a*) has shown that neoarsphenamine is 25–60 times more toxic to trypanosomes under aerobic conditions than it is when strict anaerobic conditions are observed, the change taking place within 2–5 min. of dissolving the compound; there is no difference of this kind shown in the case of mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide). It would appear, therefore, that the arsenoxides are the most highly toxic of the aromatic arsenic compounds, and much experimental evidence has accumulated which has led to the probability that this toxicity is due to the ability of the arsenoxides to react with thiol compounds.

This conclusion was anticipated by Ehrlich (1909) when he suggested that thiol groups could act as chemo-receptors for trivalent arsenic compounds. Later, Voegtlin, Dyer & Leonard (1923) postulated that the toxic action of an arsenoxide to trypanosomes is due to a condensation with thiol groups present in protoplasm, leading to a dysfunction of the system regulating cell respiration. The fact that arsenic compounds are capable of combining with organic compounds containing thiol groups (such as thiolacetic acid or thioglycollic acid) was demonstrated by Barber (1929), who showed that a condensation takes place between phenylarsenoxides and thiol-substituted compounds, giving a derivative (arylthioarsenite or thioglycollate) according to the equation

$$C_6H_5.AsO + 2RSH = C_6H_5.As\begin{matrix}SR\\SR\end{matrix} + H_2O.$$

Barber also showed that thiol compounds in sufficient concentration will reduce a phenylarsonic acid to a phenylarsenoxide,

$$C_6H_5.AsO_3H_2 + 2RSH \rightarrow C_6H_5.AsO + RSSR + 2H_2O,$$

which then combines with excess of thiol compound to give an arylthioarsenite as above. Gough & King (1930) subsequently deduced, from a scrutiny of the properties and reactions of arylthioarsenites, that these derivatives are not stable, but are slowly hydrolyzed at ordinary temperatures even in neutral solution, with liberation of small amounts of toxic arsenoxides. This supposition supported their view, that when pentavalent phenylarsonic acid derivatives are administered therapeutically in the treatment of protozoal infections, reduction takes place in the tissues to give arsenoxides, which then condense with thiol compounds of the tissues, and are 'stored' in this manner. The highly trypanocidal arsenoxides are finally set free, by hydrolysis, over an extended period of time, and are responsible for the prolonged therapeutic effect of the drug. It would appear,

therefore, that, in neutral solution, an equilibrium can exist between an arsenoxide and a thiol compound, as follows:

$$C_6H_5.AsO + 2RSH \rightleftharpoons C_6H_5.As\begin{matrix}SR\\SR\end{matrix} + H_2O.$$

If it is true that the toxicity of arsenic compounds to micro-organisms is due to a combination between arsenoxide and thiol groups, causing a breakdown in some essential metabolic process, then it would be reasonable to suppose that, in presence of excess of added thiol compound, the toxicity of the arsenic compound would be greatly reduced, since the arsenic would then be present in the system mainly in the form of arylthioarsenite, which should be non-toxic since it is incapable of further reaction with thiol groups. Such an effect was reported by Eagle (1939*b*) who discovered that, in presence of sufficient excess of thiol compounds such as cysteine, glutathione, etc., the anti-spirochaetal activity of trivalent arsenic compounds is almost completely removed; no effect of this sort was noticed when methionine was used. The large excess of thiol compound necessary to produce the effect was in harmony with the views of Gough & King (1930) upon the hydrolysis of the arylthioarsenites.

In order to obtain more information regarding the capacity of trivalent arsenic compounds to interfere with metabolic processes by virtue of their affinity for thiol groups, we considered it advisable to undertake an investigation of the manner in which arsenic compounds may influence the activities of a variety of enzyme systems. Evidence has been obtained by a number of workers that certain enzymes require, for their activity, the integrity of thiol groups in their structure (see review of earlier work by Hellerman, 1937). Toxicity of trivalent arsenoxides to enzymes may throw light on, or confirm, the thiol nature of such enzymes. The experimental results which are now reported provide evidence confirming the conclusions of earlier workers on the thiol structure of certain enzymes, and indicating for the first time the thiol structure of choline dehydrogenase. At the same time evidence will be given clearly demonstrating the reactivation by thiol compounds of enzyme systems inhibited or poisoned by arsenic compounds.

EXPERIMENTAL METHODS

Urease

Preparation and measurement of activity. A commercial (Dunning) urease tablet (25 mg.) was ground in 5 ml. saline. CO_2 gas was passed through the suspension for a few minutes at room temperature and the mixture was centrifuged. The clear supernatant fluid had high urease activity and 0·2–0·4 ml. of this fluid was used for the enzyme studies.

A manometric technique (Barcroft or Warburg) was exclusively used. The urease preparation was introduced into the main compartment of the vessel of a Warburg or Barcroft manometric apparatus in presence of 0·13 M-sodium acetate buffer, pH 5·0, at 37°. Urea (0·2 ml. of 10% solution) was placed in the side tube of the manometer vessel, and after thermal equilibrium the urea was tipped into the main vessel and readings of CO_2 output were noted at frequent intervals as the urea decomposed. The rate of CO_2 output was a measure of the activity of the urease preparation.

Additions of organic arsenic compounds and other inhibitors. These substances were added to the urease preparation in the main manometer vessel prior to the addition of urea. Thiol compounds, or other substances under investigation, were also added to the urease in the main compartment of the Warburg or Barcroft manometer vessel before addition of the urea. Any divergence from this procedure is noted in the text.

Addition of animal tissues. Experiments were carried out to determine whether the presence of animal tissues would inactivate organic arsenic compounds. Usually, known quantities of the arsenical were incubated with about 1 g. animal tissue (e.g. liver slices, or blood) at 37° for 2 hr. in presence of 0·037 M-$NaHCO_3$ solution in an atmosphere of 7% CO_2 + 93% O_2. At the end of this period 1 ml. of the supernatant was transferred to the acetate-urease mixture present in a Warburg manometric vessel. After thermal equilibrium, urea was tipped in from a side tube and the course of CO_2 evolution was followed. Control experiments in the absence of tissues were always set up.

Hydrolytic enzymes

Cholinesterase. Whole fresh rat brain (1·5 g.) was ground with sand in the presence of saline (8 ml.). The fine suspension was filtered through washed muslin and 0·5 ml. of the filtrate was used for the majority of estimations.

Activity measurements were carried out in either the Barcroft or Warburg manometric apparatus. The enzyme preparation was placed in the main compartment of a manometric vessel in presence of 0·027 M-$NaHCO_3$ solution in an atmosphere of 5% CO_2 + 95% N_2. Acetylcholine chloride (0·2 ml. of a 2% solution in $NaHCO_3$ solution) was placed in a side tube of the apparatus and this was tipped into the main vessel after thermal equilibrium had been set up. The rate of CO_2 evolution was a measure of the activity of the cholinesterase preparation.

Liver esterase. Rat-liver tissue was used as a source of liver esterase either in the form of thin, intact slices of fresh liver or as a suspension made by mincing 1 g. fresh liver in 15 ml. saline, 0·2–0·5 ml. of the suspension being usually employed. Activity was measured as with cholinesterase, with 0·2 ml. tributyrin as substrate. Control experiments without tissue were always set up.

Pancreatic lipase or esterase. Commercial pancreatin was used as a source of the enzyme and 0·5 ml. of a 1 in 100 suspension of pancreatin in saline was usually employed, with 0·2 ml. tributyrin as substrate. Activities were estimated as with cholinesterase.

Invertase. Commercial invertase was used as a source of the enzyme. The preparation (0·1 g.) was ground in 10 ml. water and the suspension was centrifuged. Of the clear centrifugate 1 ml. was usually employed. The general procedure for estimating the effects of organic arsenicals was as follows: In a series of tubes were placed 5 ml. 40% sucrose solution, 3 ml. 0·2 M-sodium acetate buffer solution, pH 5·0, and 1 ml. of either water or an aqueous solution of the organic arsenical. The mixtures were placed in a water bath at 40°, and after 30 min. 1 ml. of the invertase pre-

paration was added to each tube. At known intervals, pairs of tubes were removed from the water bath, and the contents were treated with 2 ml. of 6% trichloroacetic acid and filtered. To 8 ml. of the filtrate 2 ml. N-NaOH were added, and the mixture was transferred to a 1 dm. polarimeter tube and the optical rotation read. The course of inversion of sucrose was followed in this way and estimates were made of the effects of the organic arsenicals on the invertase activity.

Oxidation systems

The activities of these systems were measured either (*a*) under aerobic conditions by estimating the rates of O_2 uptake when tissues, or their extracts, respired in air or O_2 in a Locke 0·03 M-sodium phosphate pH 7·4 medium either in the presence or absence of organic arsenicals, or (*b*) under anaerobic conditions by means of the ferricyanide technique of Quastel & Wheatley (1938) whereby the rate of evolution of CO_2 produced from bicarbonate on reduction of ferricyanide, in an atmosphere of 5% CO_2+95% N_2, was followed.

Succinic dehydrogenase. Rat liver, or rat kidney, slices were used as sources of this enzyme when intact tissues were investigated. A purified preparation of succinic dehydrogenase made from pig heart by the method of Keilin & Hartree (1940) was also used. In Exp. 6, Table 4, 0·5 ml. of a saline suspension of minced sheep liver (1 g./5 ml. saline) was employed.

Choline dehydrogenase. This enzyme was used either in the form of 1 ml. of a saline suspension of washed, minced rat liver (1 g. liver/5 ml. saline) or as intact liver slices (25 mg. dry weight).

Cytochrome oxidase. A saline suspension (1 ml.) of minced washed rat liver was generally used.

Lactic dehydrogenase. Washed human red blood cells lysed with water (1 part of cells/3 parts water) were used as a source of this enzyme. 1 ml. of the lysed preparation, in presence of cozymase (5 mg.) and neutral NaCN (0·08 M), was usually employed.

Oxidations of glucose, lactate, or pyruvate. These oxidation systems were studied with the aid of saline suspensions of minced rat brain (1 g. whole brain/5 ml. saline), 1 ml. of the saline suspension being used.

Catalase. Minced rat liver (1 g.) was shaken with 100 ml. saline and the mixture centrifuged. The clear centrifugate served as a source of catalase, 0·5 ml. of the fluid being used.

RESULTS

Urease

Effects of a variety of organic arsenic compounds in the presence or absence of thiol compounds are shown in Tables 1 and 2 and Figs. 1 and 2.

The results show:

(1) Arsenoxides, in the form of phenylarsenoxide, mapharside(*m*-amino-*p*-hydroxyphenylarsenoxide), *p*-acetamidophenylarsenoxide, *m*-acetamido-*p*-carboxyphenylarsenoxide, and the products of condensation of thioglycollic acid with phenylarsenoxide and the reduced forms of atoxyl and tryparsamide have highly inhibitory effects on urease activity. The effects exceed those due to sodium arsenite at equivalent concentrations.

(2) Stabilarsan (the diglucoside of arsphenamine) has weakly toxic effects on urease, less than those of sodium arsenite at equivalent concentrations.

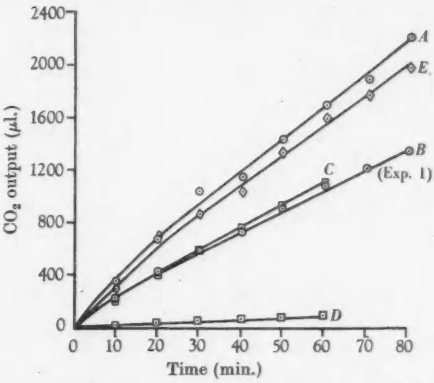


Fig. 1. Effects of arsenic compounds on urease activity at 37° and an acidity of pH 5. Exp. 1, Table 2: *A*, urease control; *B*, urease + 33 μg./ml. mapharside. Exp. 3, Table 2: *C*, urease control; *D*, urease + 67 μg./ml. phenylarsenoxide thioglycollate. Exp. 7, Table 2: *A*, urease control; *E*, urease + 670 μg./ml. tryparsamide.

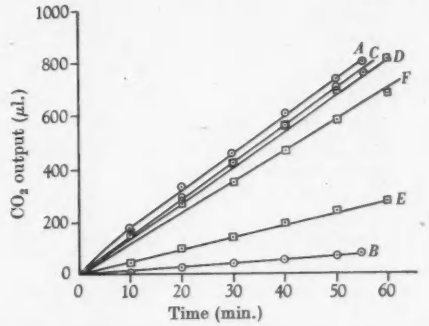
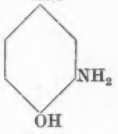
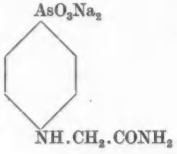
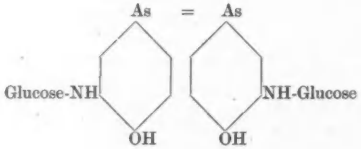


Fig. 2. Effects of arsenic compounds with and without added cysteine on urease activity at 37° and an acidity of pH 5. Exp. 14, Table 2: *A*, urease control; *B*, urease + 67 μg./ml. phenylarsenoxide thioglycollate; *C*, urease + 67 μg./ml. phenylarsenoxide thioglycollate + 2 mg./ml. cysteine. Exp. 15, Table 2: *D*, urease control; *E*, urease + 67 μg./ml. reduced tryparsamide thioglycollate; *F*, urease + 67 μg./ml. reduced tryparsamide thioglycollate + 2 mg./ml. cysteine.

(3) The presence of excess of a thiol compound, such as cysteine or glutathione, eliminates or greatly reduces the toxic effect of arsenoxides or of condensation products of arsenoxides and thioglycollic acid.

(4) Excess of NaCN does not affect the toxicity of an arsenoxide to urease.

Table 1. *Effects of arsenic compounds on urease at* 37° *and an acidity of* pH 5·0

Exp.	Arsenic compound added	Constitution	Conc. (M)	Enzyme activity (CO_2 output, µl./hr.)	Inhibition of enzyme activity (%)
1	Nil	AsO	—	1665	—
	Mapharside		$1·6 \times 10^{-4}$	1020	39
	Tryparsamide	AsO_3Na_2 	$2·1 \times 10^{-3}$	1491	10
2	Nil		—	1120	—
	Phenylarsen-oxide thio-glycollate	As(S.CH_2.COONa)(S.CH_2.COONa)	$1·8 \times 10^{-4}$	85	92
3	Nil	AsO	—	1264	—
	Phenylarsen-oxide		$4·0 \times 10^{-4}$	408	68
4	Nil		—	1308	—
	Sodium arsenite	Na_3AsO_3	$0·86 \times 10^{-3}$	876	33
	Sodium arsenite		$1·73 \times 10^{-3}$	609	54
5	Nil		—	1245	—
	Reduced atoxyl thioglycollate	As(S.CH_2.COONa)(S.CH_2.COONa)	$2·55 \times 10^{-4}$	672	47
6	Nil		—	1152	—
	Stabilarsan	As = As 	$0·97 \times 10^{-3}$	1008	13
	Stabilarsan	Glucose-NH, NH-Glucose	$2·4 \times 10^{-3}$	768	34

(5) Toxicity of an arsenoxide to urease does not increase with time, indicating the probable existence of an equilibrium between the arsenoxide and the enzyme.

(6) The presence of tissues such as liver slices, or of whole or lysed blood, diminishes toxicity of arsenoxides to urease. This is due to the fact that the effective concentration of arsenoxide is diminished by combination of the arsenical with the tissue proteins.

(7) The pentavalent arsenic compound, tryparsamide, shows little toxicity to urease.

These conclusions confirm the observations made by previous authors as to the inhibitory effects of

Table 2. *Effects of organic arsenic compounds on urease*

Exp.	Arsenic compound	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Total CO_2 output (μl.)	Inhibition of enzyme activity (%)
1	Nil	—	80	2220	—
	Mapharside	33	80	1360	39
2	Nil	—	50	633	—
	Mapharside	67	50	160	74·7
	Nil + NaCN (2 mg./ml.)	—	50	627	<1
	Mapharside + NaCN (2 mg./ml.)	67	50	163	74·2
3	Nil	—	60	1120	—
	Phenylarsenoxide thioglycollate	67	60	85	92
4	Nil	—	30	390	—
	Phenylarsenoxide thioglycollate	67	30	20	95
5	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	100	40	449	46
6	Nil	—	40	830	—
	Reduced atoxyl thioglycollate	200	40	363	56
7	Nil	—	80	2220	—
	Tryparsamide	670	80	1990	10
8	Nil	—	30	281	—
	Tryparsamide	1300	30	279	<1
9	Nil	—	15	218	—
	p-Acetamidophenylarsenoxide	33	15	195	10·6
10	Nil	—	15	215	—
	m-Acetamido-*p*-carboxyphenylarsenoxide	33	15	7	96·7
	Effects of addition of cysteine and glutathione				
11	Nil	—	55	837	—
	Nil + cysteine (2 mg./ml.)	—	55	767	8·4
	Mapharside	67	55	299	64·4
	Mapharside + cysteine (2 mg./ml.)	67	55	761	9·1
12	Nil	—	70	682	—
	Nil + cysteine (2 mg./ml.)	—	70	625	8·4
	Mapharside	67	70	216	68·3
	Mapharside + cysteine (2 mg./ml.)	67	70	591	13·3
13	Nil	—	60	852	—
	Nil + cysteine (2 mg./ml.)	—	60	867	—
	Phenylarsenoxide thioglycollate	67	60	49	94·7
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	60	612	28·2
14	Nil	—	55	809	—
	Phenylarsenoxide thioglycollate	67	55	77	90·5
	Phenylarsenoxide thioglycollate + cysteine (2 mg./ml.)	67	55	761	5·9
15	Nil	—	60	822	—
	Reduced tryparsamide thioglycollate	67	60	278	66·2
	Reduced tryparsamide thioglycollate + cysteine (2 mg./ml.)	67	60	687	16·4
16	Nil	—	45	780	—
	Nil + glutathione (2 mg./ml.)	—	45	741	5
	Mapharside	67	45	185	76·3
	Mapharside + glutathione (2 mg./ml.)	67	45	752	3·6
	Effects of addition of animal (rat) tissues				
17	Nil	—	30	694	—
	Nil + liver slices (330 mg./ml.)	—	30	642	7
	Mapharside	22	30	359	48
	Mapharside + liver slices (330 mg./ml.)	22	30	654	6
18	Nil	—	25	916	—
	Nil + whole blood (0·17 ml./ml.)	—	25	907	1
	Mapharside	33	25	464	49
	Mapharside + whole blood (0·17 ml./ml.)	33	25	821	13
19	Nil	—	30	1070	—
	Nil + lysed blood (0·17 ml./ml.)	—	30	1310	—
	Mapharside	33	30	452	58
	Mapharside + lysed blood (0·17 ml./ml.)	33	30	1390	—

Table 3. *Effects of organic arsenic compounds with and without the addition of cysteine on hydrolytic enzymes*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	CO_2 output (μl.)	Inhibition of enzyme activity (%)
		I. Cholinesterase (substrate: acetylcholine 1·3 mg./ml.)				
1	Rat brain suspension (1 ml.)	Nil	—	60	583	—
		Mapharside	500	60	369	36·7
		3-Amino-4-hydroxy-phenylarsonic acid	1300	60	502	14
2	Rat brain suspension (0·5 ml.)	Nil	—	60	373	—
		Mapharside	500	60	209	46
		3-Amino-4-hydroxy-phenylarsonic acid	600	60	347	8
3	Rat brain suspension (0·5 ml.)	Nil	—	100	459	—
		Mapharside	330	100	273	41
		Mapharside + cysteine (10 mg./ml.)	330	100	359	22
4	Rat brain suspension (0·5 ml.)	Nil	—	60	292	—
		Mapharside	330	60	170	41
		Mapharside + cysteine (10 mg./ml.)	330	60	226	22
5	Rat brain suspension (0·5 ml.)	Nil	—	70	501	—
		Nil + cysteine (10 mg./ml.)	—	70	503	—
		II. Liver esterase (substrate: tributyrin 700 μg./ml.)				
6	Rat liver slices (80 mg.)	Nil	—	25	1120	—
		Mapharside	270	25	613	45
7	Rat liver slices (50 mg.)	Nil	—	35	761	—
		Mapharside	270	35	295	61
		Tryparsamide	700	35	543	27
8	Minced liver suspension	Nil	—	20	1250	—
		Tryparsamide	700	20	555	68
9	Minced liver suspension	Nil	—	15	946	—
		Atoxyl	700	15	174	81
10	Minced liver suspension	Nil	—	40	1250	—
		Nil + cysteine (700 μg./ml.)	—	40	934	25·2
		Mapharside	330	40	306	71·2
		Mapharside + cysteine (700 μg./ml.)	330	40	680	45·6
11	Minced liver suspension	Nil	—	35	1070	—
		Nil + cysteine (800 μg./ml.)	—	35	760	29
		Mapharside	100	35	424	60·4
		Mapharside + cysteine (800 μg./ml.)	100	35	635	40·7
		III. Pancreatic lipase (substrate: tributyrin 700 μg./ml.)				
12	Pancreatin suspension	Nil	—	15	633	—
		Mapharside	330	15	628	0·8
13	Pancreatin suspension	Nil	—	20	963	—
		Tryparsamide	700	20	872	9
14	Pancreatin suspension	Nil	—	20	837	—
		Atoxyl	700	20	805	4
		IV. Invertase (substrate: 20% sucrose)			Change of optical rotation (°)	
15	Commercial yeast invertase solution	Nil	—	60	11·23	—
		Mapharside	200	60	10·78	4
16	Commercial yeast invertase solution	Nil	—	90	12·26	—
		Mapharside	200	90	12·11	1
17	Commercial yeast invertase solution	Nil	—	45	2·92	—
		Mapharside	200	45	2·85	2
18	Commercial yeast invertase solution	Nil	—	70	3·82	—
		Mapharside	200	70	3·81	0

substances (e.g. heavy metals, quinones, etc.) which react with thiol groups (Quastel, 1933; Perlzweig, 1932; Hellerman, Perkins & Clarke, 1933; Sumner & Poland, 1933; Smythe, 1936; Weiss, 1937) and support the conclusion that urease depends for its activity on the presence of thiol groups in its structure.

Pentavalent arsenic compounds become appreciably toxic only when reduction to the trivalent arsenoxide occurs, and this toxicity may be eliminated either by combination with thiol compounds, which must be in excess to prevent too great a dissociation of the easily reversible arsenoxide-thiol complex, or by combination with tissue proteins.

Hydrolytic enzymes

Cholinesterase. Typical results showing the effects of arsenicals on cholinesterase are given in Table 3 and Fig. 3. These results demonstrate:

(1) Cholinesterase is inhibited by the trivalent mapharside but not, or only feebly, by the pentavalent 3-amino-4-hydroxyphenylarsonic acid.

(2) The effect of the arsenoxide on the enzyme does not increase with time, showing the existence of an equilibrium between arsenical and enzyme.

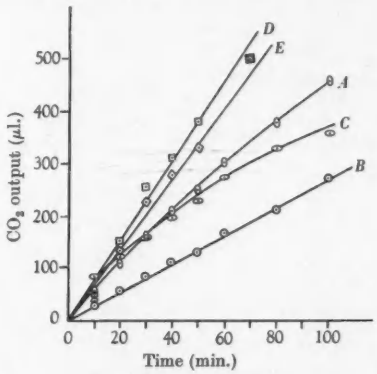


Fig. 3. Effects of arsenic compounds with and without added cysteine on cholinesterase at 37°. Exp. 3, Table 3: *A*, cholinesterase control; *B*, cholinesterase + 330 μg./ml. mapharside; *C*, cholinesterase + 330 μg./ml. mapharside + 10 mg./ml. cysteine. Exp. 5, Table 3: *D*, cholinesterase control; *E*, cholinesterase + 10 mg./ml. cysteine.

(3) The inhibitory action of the arsenoxide is greatly diminished by the presence of excess of cysteine.

The facts support the view that cholinesterase is a thiol enzyme (Nachmansohn & Lederer, 1939).

Liver esterase. Results obtained with this enzyme are shown in Table 3. Liver esterase is exceptional, among the enzymes which have been investigated, in being inhibited by pentavalent as well as by trivalent arsenic compounds. Thus tryparsamide and atoxyl exert toxic effects which are comparable with those due to mapharside. The toxic effect of atoxyl has already been noted by Jowett & Quastel (1935). The inhibitory effects of mapharside are not greatly diminished, or nullified, by the addition of excess of cysteine. A diminution in toxicity does take place, but it is difficult to assess, since cysteine alone exerts inhibitory effects on the activity of the liver esterase preparations we have used.

The facts we have obtained indicate the sensitivity of the enzyme preparation to the presence of arsenic whether this is in the trivalent or pentavalent condition. It would be wise to suspend judgement as to the thiol nature of the enzyme until a purified preparation of liver esterase has been investigated.

Pancreatic esterase. This enzyme preparation, in contrast to that of liver esterase, showed little or no sensitivity to trivalent or pentavalent organic arsenicals, under the conditions of our experiments.

Invertase. This enzyme is not attacked by mapharside at the concentrations tried (see Table 3), and is therefore unlikely to be a thiol enzyme.

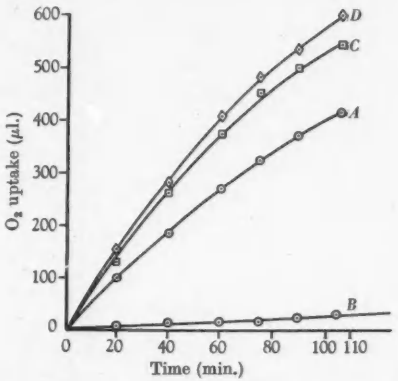


Fig. 4. Effects of mapharside, with and without added glutathione, on succinic dehydrogenase activity. Exp. 4, Table 4: *A*, succinic dehydrogenase control; *B*, succinic dehydrogenase + 2·7 μg./ml. mapharside; *C*, succinic dehydrogenase + 700 μg./ml. glutathione; *D*, succinic dehydrogenase + 2·7 μg./ml. mapharside + 700 μg./ml. glutathione.

Oxidation systems

Succinic dehydrogenase. Results in Table 4 and Fig. 4 show the highly inhibitory effects of mapharside on the activity of succinic dehydrogenase and the lack of toxic action of tryparsamide. The poisoning effect of the trivalent arsenical is nullified by the presence of an excess of glutathione. These

Table 4. *Effects of organic arsenic compounds on oxidizing enzymes in presence and absence of thiol compounds*

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	O_2 uptake (μl.)	Inhibition of enzyme activity (%)
		I. Succinic dehydrogenase (substrate: sodium succinate M/30)				
1	Rat liver slices (100 mg.)	Nil	—	30	596	—
		Mapharside	70	30	269	58
2	Rat kidney slices (100 mg.)	Nil	—	130	581	—
		Mapharside	133	130	192	67
3	Rat kidney slices (100 mg.)	Nil	—	70	539	—
		Tryparsamide	7000	70	536	0
4	Pig heart extract (0·5 ml.)	Nil	—	105	414	—
		Mapharside	2·7	105	28	93
		Nil + glutathione (700 μg./ml.)	—	105	544	—
		Mapharside + glutathione (700 μg./ml.)	2·7	105	597	0
5	Pig heart extract (0·5 ml.)	Nil	—	60	470	—
		Mapharside	2·7	60	167	64
		Nil + glutathione (700 μg./ml.)	—	60	544	—
		Mapharside + glutathione (700 μg./ml.)	2·7	60	535	0
					Anaerobic CO_2 output (μl.)	
6	Sheep liver extract (0·5 ml.)	Nil	—	30	599	—
		Mapharside	170	30	137	77
7	Rat liver slices (100 mg.)	Nil	—	60	917	—
		Mapharside	133	60	264	71
		II. Choline dehydrogenase (substrate: choline M/30)			O_2 uptake (μl.)	
8	Rat liver suspension (1 ml.)	Nil	—	40	388	—
		Reduced tryparsamide thioglycollate	170	40	212	45
		Reduced tryparsamide thioglycollate	330	40	115	70
9	Rat liver suspension (1 ml.)	Nil	—	60	626	—
		Reduced tryparsamide thioglycollate	230	60	249	60
		Tryparsamide	1300	60	586	6
10	Rat liver suspension (1 ml.)	Nil	—	60	228	—
		Mapharside	200	60	65	71
		Mapharside + cysteine (5 mg./ml.)	200	60	223	0
					Anaerobic CO_2 output (μl.)	
11	Rat liver slices (25 mg. dried tissue)	Nil	—	45	791	—
		Mapharside	130	45	388	50
12	Rat liver suspension (1 ml.)	Nil	—	60	707	—
		Mapharside	130	60	463	34
13	Rat liver slices (25 mg. dried tissue)	Nil	—	60	640	—
		Mapharside	170	60	212	67
		Mapharside + cysteine (5 mg./ml.)	170	60	539	16
		III. Cytochrome oxidase (substrate: *p*-phenylenediamine 3·3 mg./ml.)			O_2 uptake (μl.)	
14	Rat liver suspension (1 ml.)	Nil	—	40	472	—
		Mapharside	133	40	480	0
		Mapharside	330	40	466	1

Table 4 (*cont.*)

Exp.	Source of enzyme	Arsenic compound added	Conc. of arsenic compound (μg./ml.)	Duration of exp. (min.)	Anaerobic CO_2 output (μl.)	Inhibition of enzyme activity (%)
		IV. Lactic dehydrogenase (substrate: sodium lactate 16 mg./ml.)				
15	Lysed human red blood cells (1 blood : 3 water) (1 ml.) + cozymase + NaCN (neutral, M/12)	Nil	—	60	639	—
		Mapharside	330	60	598	6
		V. Glucose oxidation (substrate: glucose 0·01 M)				
18	Rat brain suspension (1 ml.)	Nil	—	150	349	—
		Mapharside	170	150	176	50
19	Rat brain suspension (1 ml.)	Nil	—	180	476	—
		Mapharside	170	180	99	79
		Tryparsamide	1700	180	650	0
20	Rat brain suspension (1 ml.)	Nil	—	120	583	—
		Mapharside	170	120	264	55
		Mapharside + cysteine (3·3 mg./ml.)	170	120	567	3
21	Rat brain suspension (1 ml.)	Nil	—	180	317	—
		3-Acetamido-4-carboxy-phenylarsenoxide	33	180	241	24
		3-Acetamido-4-carboxy-phenylarsenoxide + cysteine (660 μg./ml.)	33	180	319	0
		VI. Pyruvate oxidation (substrate: sodium pyruvate 0·01 M)				
22	Rat brain suspension (1 ml.)	Nil	—	150	360	—
		Mapharside	200	150	95	74
23	Rat brain suspension (1 ml.)	Nil	—	180	634	—
		Nil + glutathione (5 mg./ml.)	—	180	596	6
		Mapharside	200	180	12	98
		Mapharside + glutathione (5 mg./ml.)	200	180	420	33
24	Rat brain slices	Nil	—	110	185	—
		Mapharside	200	110	74	60
		VII. Lactate oxidation (substrate: sodium lactate 0·02 M)				
25	Rat brain suspension (1 ml.)	Nil	—	60	200	—
		Mapharside	133	60	3	98
		Mapharside	67	60	27	86
26	Rat brain suspension (1 ml.)	Nil	—	150	463	—
		Nil + glutathione (330 μg./ml.)	—	150	452	2
		Mapharside	17	150	25	94
		Mapharside + glutathione (330 μg./ml.)	17	150	363	21
		VIII. Catalase (substrate: H_2O_2, 0·03%)			O_2 output (μl.)	
27	Rat liver extract (0·5 ml.)	Nil	—	40	621	—
		Mapharside	133	40	614	1
		Mapharside	330	40	556	10

facts point to the thiol nature of succinic dehydrogenase, confirming the observations of Hopkins & Morgan (1938), Hopkins, Morgan & Lutwak-Mann (1938) and of Keilin & Hartree (1940). The greater the purity of the enzyme preparation the greater is the toxicity secured by a given concentration of arsenoxide. This is due to the purified preparation containing smaller quantities of tissue proteins which compete with the enzyme for the arsenical.

Choline dehydrogenase. This enzyme (for details of properties and estimation see Mann & Quastel, 1937; Bernheim & Webster, 1937; Mann, Woodward & Quastel, 1938) is highly sensitive to such trivalent organic arsenic compounds as mapharside and reduced tryparsamide thioglycollate. The pentavalent tryparsamide, however, does not inhibit the enzyme. The presence of cysteine, in excess, reverses the inhibitory action of mapharside on choline

Table 5. *Effects of various inhibitors on choline dehydrogenase*

(Substrate: choline 0·033 M)

Exp.	Source of enzyme	Inhibitor	Conc. of inhibitor (M)	Duration of exp. (min.)	O_2 uptake (μl.)	Inhibition of enzyme activity (%)
1	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	167	—
		Sodium tetrathionate (choline absent)	2×10^{-4}	60	102	—
		Nil	—	60	621	—
		Sodium tetrathionate	2×10^{-4}	60	382	38
2	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	75	—
		Sodium tetrathionate (choline absent)	6×10^{-4}	60	43	—
		Nil	—	60	549	—
		Sodium tetrathionate	6×10^{-4}	60	80	93
3	Saline suspension of washed minced rat liver	Nil (choline absent)	—	60	89	—
		Alloxan (choline absent)	0·22	60	31	—
		Nil	—	60	508	—
		Alloxan	0·22	60	107	82
4	Saline suspension of washed minced rat liver	Nil	—	60	482	—
		Alloxan	0·022	60	90	82
5	Saline suspension of washed minced rat liver	Nil (choline absent)	—	150	63	—
		Nil	—	150	317	—
		Oxidized glutathione	0·05	150	204	44

dehydrogenase. Results supporting these conclusions are shown in Table 4 and Figs. 8 and 9. These results lead to the view that choline dehydrogenase is a thiol enzyme. This conclusion is supported by the fact that the following compounds also inhibit choline dehydrogenase, viz. sodium tetrathionate, alloxan, and oxidized glutathione. Typical results are shown in Table 5. These substances, as shown by Hopkins & Morgan (1938) and by Keilin & Hartree (1940), have inhibitory effects on succinic dehydrogenase, presumably by oxidation of the thiol groups of the enzyme.

Pyruvic oxidase. The oxidation of pyruvic acid by minced rat brain is very greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione (Table 4, and Fig. 6). These facts point to the thiol nature of pyruvic oxidase, already suggested by Peters (1937) and by experiments of Barron (1936) on pyruvate oxidation of gonococci.

Glucose and lactate oxidation. The respiration of minced rat brain in presence of either glucose or sodium lactate is very greatly inhibited by mapharside and by 3-acetamido-4-carboxyphenylarsenoxide, the inhibitions being greatly reduced or eliminated by the presence of excess cysteine. These results are shown in Table 4 and Figs. 5 and 7. The pentavalent tryparsamide has no inhibitory effect on oxygen uptake by brain in presence of glucose under the conditions of our experiments. We have noted that in all experiments on the respiration of brain tissue in presence of glucose or of lactate, there is an accumulation of a ketonic acid, probably pyruvic acid, in the medium when the trivalent organic arsenical is also present. Doubtless the large inhibitory effects of the trivalent organic arsenicals on brain respiration in presence of glucose or lactate are due partly to the blocking of the respiratory chain at the pyruvic enzyme stage.

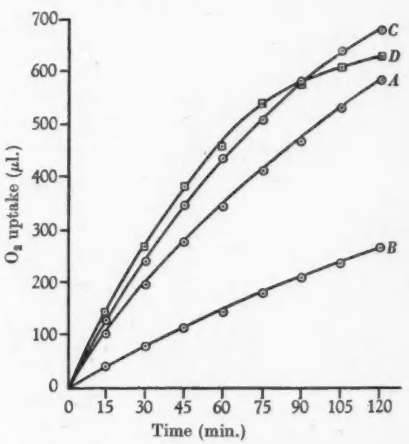


Fig. 5. Effects of mapharside with and without added cysteine, on glucose oxidation at 37°. Exp. 20, Table 4: *A*, glucose oxidation system control; *B*, glucose oxidation system + 170 μg./ml. mapharside; *C*, glucose oxidation system + 3·3 mg./ml. cysteine; *D*, glucose oxidation system + 170 μg./ml. mapharside + 3·3 mg./ml. cysteine.

Lactic dehydrogenase. Results given in Table 4, Exp. 15, show that lactic dehydrogenase, examined by the anaerobic technique, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

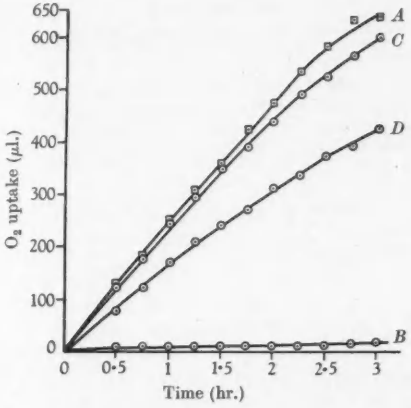


Fig. 6. Effects of mapharside, with and without added glutathione, on pyruvic oxidase activity at 37°. Exp. 23, Table 4: *A*, pyruvic oxidase control; *B*, pyruvic oxidase + 200 μg./ml. mapharside; *C*, pyruvic oxidase + 5 mg./ml. glutathione; *D*, pyruvic oxidase + 200 μg./ml. mapharside + 5 mg./ml. glutathione.

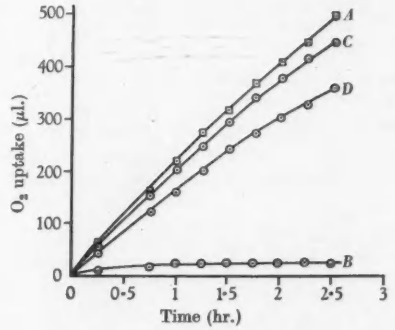


Fig. 7. Effects of mapharside and glutathione on lactic oxidation system at 37°. Exp. 26, Table 4: *A*, lactate oxidation system control; *B*, lactate oxidation system + 17 μg./ml. mapharside; *C*, lactate oxidation system + 330 μg./ml. glutathione; *D*, lactate oxidation system + 17 μg./ml. mapharside + 330 μg./ml. glutathione.

Cytochrome oxidase and catalase. Neither of these enzymes is affected by mapharside under the conditions of our experiments and it is unlikely that they are thiol enzymes.

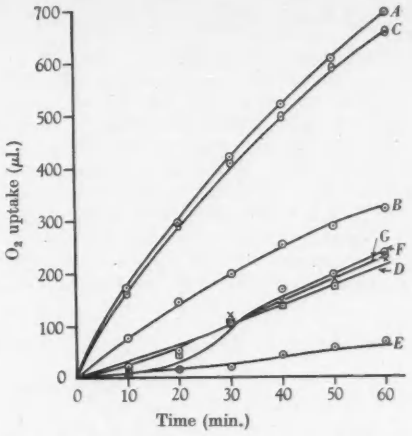


Fig. 8. Effects of arsenic compounds, with and without added cysteine, on choline dehydrogenase activity at 37°. Exp. 9, Table 4: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 230 μg./ml. reduced tryparsamide thioglycollate; *C*, choline dehydrogenase + 1300 μg./ml. tryparsamide. Exp. 10, Table 4: *D*, choline dehydrogenase control; *E*, choline dehydrogenase + 200 μg./ml. mapharside; *F*, choline dehydrogenase + 200 μg./ml. mapharside + 5 mg./ml. cysteine (cysteine added at same time as mapharside); *G*, choline dehydrogenase + 200 μg./ml. mapharside + 5 mg./ml. cysteine (cysteine added after 2 hr. incubation of enzyme and mapharside).

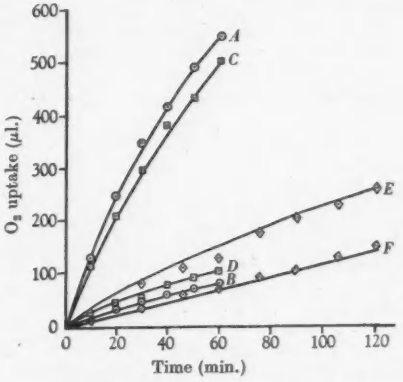


Fig. 9. Effects of thiol inhibitors on choline dehydrogenase activity at 37°. Exp. 2, Table 5: *A*, choline dehydrogenase control; *B*, choline dehydrogenase + 6×10^{-4} M-sodium tetrathionate. Exp. 4, Table 5: *C*, choline dehydrogenase control; *D*, choline dehydrogenase + 0·022 M-alloxan. Exp. 5, Table 5: *E*, choline dehydrogenase control; *F*, choline dehydrogenase + 0·05 M-oxidized glutathione.

Protection of enzymes by their substrates

Experiments were carried out on a few of the enzymes, which were shown to be inhibited by arsenoxides, to determine whether the enzymes could be protected from inactivation by their substrates. The enzymes which were examined in this way were urease, succinic dehydrogenase and choline dehydrogenase. The activities of the enzymes were measured in the manner already described, except that the inhibitor was added before the substrate. Controls were also performed, in which the substrate was added last. Results are shown in Table 6.

Table 6. *Effects of the presence of substrates on inactivation of enzymes by mapharside*

(A) Urease (final concentration of urea 16%)

Final concentration of mapharside (μg./ml.)	Conditions of experiment	CO_2 output (μl.)	Inhibition of enzyme added (%)
0	Urea added last	1749	—
33	Urea added last	960	45
0	Urea added before mapharside	1791	—
33	Urea added before mapharside	1131	37

(B) Succinic dehydrogenase (conc. of substrate M/30)

Source of enzyme	Wt. of wet tissue (mg.)	Conc. of mapharside (μg./ml.)	Conditions of experiment	O_2 uptake (μl.)	Inhibition of activity (%)
Rat kidney slices	100	0	Succinate added last	393	—
	100	33	Succinate added last	225	44
	100	0	Succinate added before mapharside	399	—
	100	33	Succinate added before mapharside	327	19
Pigeon kidney slices	100	0	Succinate added last	334	—
	100	33	Succinate added last	204	39
	100	0	Succinate added before mapharside	322	—
	100	33	Succinate added before mapharside	282	13

(C) Choline dehydrogenase

Rat liver extract (1 in 4)	1 ml. extract	0	—	368	—
	1 ml. extract	133	Choline added last	194	48
	1 ml. extract	133	Choline added before mapharside	281	32

Urease is obviously not protected by its substrate (see also Quastel, 1932). Succinic dehydrogenase is protected to some extent by succinate when the concentration of mapharside is low, and choline dehydrogenase is also partially protected by its substrate.

Reduction of tryparsamide and atoxyl by thiol compounds and the effects of the reduction products on urease

It is already known (Levaditi, Andrews & Mann, 1928) that atoxyl in presence of glutathione and of tissue extracts becomes strongly trypanocidal, an effect attributed to the reduction of the pentavalent arsenical, by thiol compounds, to the active trivalent arsenoxide. This phenomenon has been confirmed with a variety of organic pentavalent arsenic compounds including tryparsamide in the presence of glutathione or whole blood (Hawking & Quastel, 1936).

It became of interest to see whether the reduction products of tryparsamide and atoxyl, due to incubation with thiol compounds, would inactivate urease.

The arsenical (0·1 g.) was dissolved in 5 ml. water to which 10 mg. cysteine was added. The solution was neutralized, placed in a vacuum tube from which air was withdrawn, and the tube was incubated at 37°. The tube was then opened and various quantities of the solutions were added to urease in a Warburg manometric vessel, from the side tube of which urea was tipped after thermal equilibrium.

Typical results are shown in Table 7, which also shows the effects obtained when excess of the thiol compound was added to the urease system. The results show that both atoxyl and tryparsamide may be transformed into highly toxic products by incubation with glutathione, cysteine or thioglycollate. Moreover the toxicities are diminished or eliminated by having an excess of glutathione present with the urease. The facts indicate that urease may be used to detect the formation of trivalent arsenoxide by reduction of pentavalent arsenicals by thiol compounds.

Table 7. *Inhibition of urease activity by pentavalent organic arsenic compounds incubated with thiol compounds*

Exp.	Nature and conditions of incubation mixture	Amount of incubation mixture added to urease (ml.)	Duration of exp. with urease (min.)	CO_2 output (μl.)	Inhibition of urease activity (%)
1	—	Nil	35	612	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	1	35	44	93
2	—	Nil	40	717	—
	Atoxyl (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	104	86
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 72 hr. at 37°	0·5	40	81	89
3	—	Nil	40	471	—
	Tryparsamide (20 mg./ml.) + Na thioglycollate (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	65
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) finally added on transference to urease	0·1	40	377	20
4	—	Nil	40	640	—
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°	0·1	40	167	74
	Tryparsamide (20 mg./ml.) + cysteine (2 mg./ml.) incubated 19 hr. at 37°. Excess glutathione (10 mg.) added on transference to urease	0·1	40	547	7
5	—	Nil	30	457	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	30	271	40
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°. Excess glutathione (5 mg.) added on transference to urease	0·5	30	384	16
6	—	Nil	50	323	—
	Tryparsamide (20 mg./ml.) + glutathione (2 mg./ml.) incubated 20 hr. at 37°	0·5	50	195	40

DISCUSSION

Our experimental results justify the view that trivalent organic arsenoxides may be employed for the detection of thiol enzyme systems. Four enzymes, namely urease, succinic dehydrogenase, cholinesterase, and pyruvic oxidase, for which there was prior evidence that thiol groups are important for their activities, are highly sensitive to phenylarsenoxide and its derivatives. Enzymes, however, for which there has been no evidence that thiol groups are essential for their activities, viz. lactic dehydrogenase, cytochrome oxidase, catalase and invertase, are relatively unaffected by the presence of organic arsenoxides.

Pentavalent organic arsenic compounds such as tryparsamide or atoxyl do not affect enzymes (except in the case of liver esterase), but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

The organic arsenoxides probably establish an equilibrium with the thiol enzymes, as their toxicities do not increase with time. These toxicities may be diminished or eliminated by the presence of an excess of thiol compounds which convert the arsenoxides into inert condensation products whose dissociations are smaller the greater the excess of free thiol groups.

Choline dehydrogenase is shown, by its high sensitivity to organic arsenoxide, or to the condensation products of these substances with thioglycollic acid, which dissociate freely in absence of excess thiol compounds, to be a thiol enzyme. This is confirmed by showing the enzyme to be sensitive also to the presence of sodium tetrathionate, alloxan or oxidized glutathione, molecules which bring about the oxidation of SH groups. This enzyme, too, probably forms an equilibrium with organic arsenoxide, and the enzyme inactivated by this means may be reactivated by the presence of excess thiol groups, as is also the case with the other thiol enzymes investigated (succinic dehydrogenase, urease, pyruvic oxidase, cholinesterase).

It is obvious that these results have a bearing on the therapeutic properties of organic arsenicals. The reduction of these substances by tissue constituents and the removal of the resultant arsenoxides by

combination with inert proteins, or their transformation into inert condensation products by local high concentrations of SH bodies, are important factors to be considered in the problems of arsenic therapy.

SUMMARY

1. A description is given of experiments carried out during 1938, 1939 and early 1940, details of which have not hitherto been published, on the effects of organic trivalent and pentavalent arsenic compounds on enzyme systems.

2. Urease is highly sensitive to trivalent arsenoxides such as phenylarsenoxide, *m*-amino-*p*-hydroxyphenylarsenoxide (mapharside), *p*-acetamidophenylarsenoxide, and *m*-acetamido-*p*-carboxyphenylarsenoxide. It is also inhibited by the products of condensation of thioglycollate with phenylarsenoxide and with the reduced forms of atoxyl and tryparsamide. The effects are generally greater than those due to sodium arsenite at equivalent concentrations. Urease is also sensitive to stabilarsan, the diglucoside of arsphenamine, but this substance is less toxic than sodium arsenite at equivalent concentrations. The toxicity of arsenoxides may be reduced or eliminated by the presence of excess of thiol compounds such as cysteine or glutathione, or by the presence of proteins which compete with urease for the arsenoxides. An equilibrium probably exists between the arsenoxide and the enzyme. Pentavalent arsenic compounds such as atoxyl or tryparsamide show little toxicity to urease. The results conform with the view that urease is a thiol enzyme.

3. The reaction of cholinesterase with arsenicals also supports the conclusion that this enzyme is a thiol enzyme.

4. Choline dehydrogenase is shown to be a thiol enzyme, since it is inhibited by trivalent arsenoxides, the inhibition being neutralized by the presence of excess of thiol compounds. It is also inhibited by sodium tetrathionate, alloxan, and oxidized glutathione.

5. Pyruvic oxidase is greatly inhibited by mapharside, the inhibition being suppressed by the presence of excess of glutathione. These results point to the thiol nature of the enzyme. The oxidation systems of lactate and of glucose, in brain tissue, are also inhibited by trivalent arsenoxide, the effects being reversed by the presence of excess thiol compounds. Lactic dehydrogenase, however, is unaffected by mapharside and is therefore unlikely to be a thiol enzyme.

6. Cytochrome oxidase, catalase and invertase are relatively unaffected by the presence of organic arsenoxides.

7. Pentavalent organic arsenic compounds do not affect the enzymes investigated, except in the case of liver esterase, but they become active poisons to the thiol enzymes when they are reduced to the trivalent arsenoxides by the presence of thiol compounds.

8. The thiol enzymes apparently form reversible equilibria with organic trivalent arsenoxides.

9. Whereas succinate protects its enzyme from inactivation by a trivalent arsenoxide and choline likewise shows some protection to its enzyme, urea fails to afford any protection to urease.

10. Mapharside is recommended as a useful organic trivalent arsenical for the detection of thiol enzymes.

We are greatly indebted to Dr H. King, F.R.S., for his gift of organic trivalent arsenoxides and their condensation products with thioglycollic acid.

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The Metabolism of Sulphonamides

5. A STUDY OF THE OXIDATION AND ACETYLATION OF SULPHONAMIDE DRUGS AND RELATED COMPOUNDS IN THE RABBIT

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It has now been established that both the simple and heterocyclic sulphonamides may undergo both acetylation and oxidation in the body (cf. Williams, 1945, 1946, 1947; Dobson & Williams, 1946; Stubbs & Williams, 1947; Bray, Neale & Thorpe, 1946; Gilligan, 1945; Scudi & Silber, 1944; Weber, Lalich & Major, 1943). In the simpler sulphonamides, such as the *o*-, *m*- and *p*-aminobenzenesulphonamides, oxidation occurs in the benzene ring, whereas in the *N'*-substituted sulphonamides, sulphapyridine and sulphaquinoxaline (Bray *et al.* 1946; Weber *et al.* 1943; Scudi & Silber, 1944), oxidation occurs in the heterocyclic ring and acetylation in the benzene ring.

The object of the present work was to find out whether *in vivo* oxidation and acetylation were related. These two processes could occur in four ways: (1) acetylation followed by oxidation of the acetylated molecule, (2) oxidation followed by acetylation of the oxidized molecule, (3) simultaneous oxidation and acetylation of the same molecule, and (4) oxidation and acetylation as separate reactions involving different molecules of the compound:

(1) $R\text{H}.\text{NH}_2 \rightarrow R\text{H}.\text{NHAc} \rightarrow R(\text{OH})\text{NHAc}$.
(2) $R\text{H}.\text{NH}_2 \rightarrow R(\text{OH})\text{NH}_2 \rightarrow R(\text{OH})\text{NHAc}$.
(3) $R\text{H}.\text{NH}_2 \rightarrow R(\text{OH})\text{NHAc}$.
(4) $2R\text{H}.\text{NH}_2 \rightarrow R\text{H}.\text{NHAc} + R(\text{OH})\text{NH}_2$.

It was suggested by Dobson & Williams (1946) that, in the case of metanilamide, oxidation does not occur in the molecule undergoing acetylation *in vivo*. Furthermore, orthanilamide is highly oxidized but hardly acetylated (Stubbs & Williams, 1947), whereas sulphanilamide is highly acetylated but only slightly oxidized in the rabbit (Shelswell & Williams, 1940; Williams, 1945, 1946). There are, therefore, suggestions that the fourth of the above mechanisms is plausible.

In the present work evidence that the compound studied has been oxidized is based on glucuronic acid and ethereal-sulphate determinations. If a compound devoid of hydroxyl groups is fed to an animal and gives rise to the excretion of increased amounts of glucuronic acid and/or ethereal sulphate, it is reasonable to conclude that the compound has been oxidized. One has, however, to bear in mind that oxidation could take place without the formation of *O*-conjugates and in such cases proof of oxidation requires the isolation of the oxidation product (cf. Ambamide; Hartles & Williams, 1947). Such cases, however, are likely to be uncommon in the series of compounds concerned here.

METHODS

Animals

Rabbits (giant chinchilla) were used throughout this investigation. They were maintained on a constant diet of 50 g. of Lever's cubes, with water *ad lib.* All drugs were administered with water by stomach tube.

Analytical methods

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944). Sanchez (1945) has observed a colour reaction between furfural and sulphadiazine. This fact had to be considered when glucuronic acid was determined in the presence of pyrimidine compounds. Glucuronic acid was therefore estimated in pure glucurone solutions in the presence of sulphadiazine and sulphapyrazine and their derivatives. None of these drugs had any influence on the recovery of glucuronic acid.

Ethereal sulphate was determined by the Folin gravimetric method (cf. Williams, 1938).

Free and combined diazotizable amino groups were determined by the colorimetric method of Bratton & Marshall (1939) using *N*-(1-naphthyl)-ethylenediamine as coupling agent. Colour intensities were measured by means of a Spekker photoelectric absorptiometer with suitable filters (in most cases Ilford no. 604 green or no. 605 yellow green). Standard curves were constructed for each drug studied.

Free amino groups were determined directly on urine appropriately diluted. Total amino groups were determined after acid hydrolysis. The concentration of acid and duration of hydrolysis were varied to suit the compound under investigation. The extent of acetylation was calculated as the difference between the values for free and total diazotizable amino groups. This calculation, however, is not valid in every case, particularly in those cases where oxidation is extensive (see Dobson & Williams, 1946).

RESULTS

Sulphadiazine, sulphapyrazine and their derivatives

The collected results for these compounds are given in Table 1. In Table 2 a complete set of results is given for sulphamezathine in order to illustrate the type of data on which the average figures in Table 1 and other tables are based.

Table 1. *The oxidation and acetylation of sulphadiazine and sulphapyrazine and their derivatives in the rabbit*

Compound fed	Dose (g./kg.)	Drug excreted: Total (% of dose)	Drug excreted: Free (% of dose)	Acetylated (% of dose)	Acetylated (% of total)	Oxidized (% of dose): Glucuronide	Oxidized (% of dose): Ethereal sulphate
Free drugs:							
Sulphadiazine	0·2	74	16	58	78	—	0
	0·5	82	26	56	68	5	0
	1·0	92	21	72	78	0	—
Sulphamerazine	0·2	76	7	69	91	—	0
	0·5	81	9	72	89	8	0
Sulphamezathine	0·2	87	10	77	88	15	0
	0·5	90	16	74	82	16	0
Sulphapyrazine	0·2	74	10	64	86	—	—
	0·5	83	10	73	88	0	—
Acetylated drugs:							
Acetylsulphadiazine	0·45	75	0·5	75	100	0	—
Acetylsulphamerazine	0·4	71	0·5	71	100	0	—
Acetylsulphamezathine	0·5	102	0·5	102	100	0	—
Acetylsulphapyrazine	0·5	79	1·0	78	99	0	—
Oxidized drugs:							
SDOH*	0·2	90	78	12	13	0	—
SM.COOH	0·2	81	73	8	10	0	—
AcSM.COOH	0·2	82	1	81	99	0	—

* SDOH is 2-sulphanilamido-4-hydroxypyrimidine (hydroxysulphadiazine); SM.COOH is 2-sulphanilamido-4-carboxy-6-methylpyrimidine; AcSM.COOH is 2-(N^4-acetylsulphanilamido)-4-carboxy-6-methylpyrimidine.

Table 2. *The excretion of metabolites of sulphamezathine in the rabbit*

(Dose 0·5 g./kg.)

Rabbit no.	Amount of drug recovered (% of dose): Total	Free	Acetylated	As glucuronide*	As ethereal sulphate
72	64	8	56	—	—
68	86	21	65	—	—
71	96	15	81	15	—
78	94	16	78	14	—
76	91	14	76	10	—
67	93	9	84	16	—
78	98	21	77	12	—
81	95	22	72	16	—
60	—	—	—	—	0
66	—	—	—	—	0
67	—	—	—	—	0

* It is assumed that one molecule of the drug gives rise to one molecule of glucuronic acid.

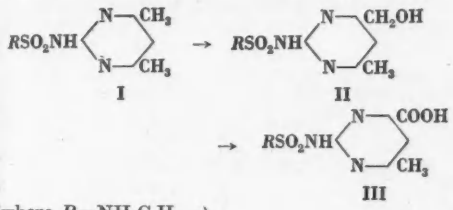
Table 1 shows that neither sulphadiazine (m.p. 252°) nor sulphapyrazine (m.p. 259°) undergoes any oxidation in the rabbit, whereas the substituted sulphadiazines, sulphamerazine (m.p. 236°) and sulphamezathine (m.p. 198°) both give rise to increased outputs of glucuronic acid. None of these compounds gives rise to ethereal sulphates. Similar results were obtained by Gilligan (1945) on hospital patients undergoing treatment with these four drugs. Some of Gilligan's results indicate an oxidation up to 68 % with sulphamezathine, but none at all with sulphadiazine and sulphapyrazine.

All four compounds are highly acetylated, although sulphadiazine is less acetylated than the other three. When the acetylated drugs are fed they are excreted unchanged, being neither deacetylated nor oxidized. Previous work on N^4-acetylsulphonamides had shown that they were not deacetylated in man (e.g. Strauss, Lowell, Taylor & Finland, 1941).

A comparison of the figures (in Table 1) for the amounts of sulphamerazine and sulphamezathine excreted free, with those for the amounts oxidized, shows that they correspond. These figures can be interpreted as meaning that it is the free and not the acetylated drugs that are oxidized. This interpretation is supported by the finding that neither N^4-acetylsulphamerazine nor N^4-acetylsulphamezathine is oxidized. These results indicate that

oxidation and acetylation are separate reactions involving different molecules of the drug. If, therefore, acetylation of these two drugs could be suppressed, the extent of their oxidation would be expected to increase.

Several unsuccessful attempts were made to isolate and identify the glucuronide in sulphamezathine urine. Nevertheless, a consideration of Table 1 does allow us to make a suggestion concerning the possible nature of this oxidation product. The main difference between sulphadiazine on the one hand, and sulphamerazine and sulphamezathine on the other, lies in the presence of substituent methyl groups in the pyrimidine rings of the latter. It is possible that these methyl groups may undergo oxidation. In the case of sulphamezathine (I) oxidation could proceed as follows:

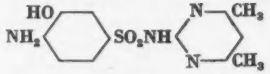


I II III

(where $R = NH_2C_6H_4$—).

Compound (III) (2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid, m.p. 253°) was therefore prepared (see below) and fed to rabbits. This compound, however, did not form a glucuronide and was only slightly acetylated (8%; see Table 1). Its N^4-acetyl derivative, m.p. 260° (prepared according to Rose & Swain, 1945), like other acetylated sulphonamides, is excreted unchanged. If this acid formed a glucuronide it would be expected to be an ester glucuronide, i.e. a substance which would reduce Fehling's solution (cf. benzoylglucuronide). Sulphamezathine urine, however, does not reduce Fehling's solution, and is therefore unlikely to contain a glucuronide of this type. The carboxylic acid (III) forms a characteristically insoluble copper salt (see below), but such a copper salt could not be isolated from sulphamezathine urine appropriately treated.

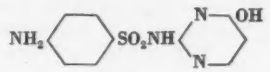
Another possible oxidation product of sulphamezathine is 2-(3′-hydroxysulphanilamido)-4:6-dimethylpyrimidine (IV), the analogue of 3-hydroxysulphanilamide (cf. Williams, 1946, 1947). Such a compound, however, would be expected to form an



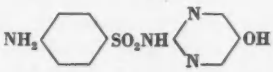
IV

ethereal sulphate. But there is no detectable increase in ethereal sulphate output after feeding sulphamezathine. Compound (IV) is therefore a possible but not probable oxidation product. Since sulphamezathine gives rise to a non-reducing glucuronide with no ethereal sulphate, a compound such as 2-sulphanilamido-4-hydroxymethyl-6-methylpyrimidine (II) would be an oxidation product which would fit the evidence obtained so far. We have not been able to synthesize this compound.

Table 2 also includes 4-hydroxysulphadiazine (V). Theoretical considerations of the electron distribution on the pyrimidine ring indicate that position 5

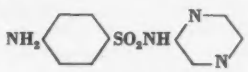


V



VI

is the most likely point of attack. If sulphadiazine were oxidized *in vivo* then 5-hydroxysulphadiazine (VI) would be expected to be formed. We have not synthesized (VI), but we prepared (V), m.p. 280°, according to Ganapathi, Deliwala & Shirsat (1942). On feeding (V) to rabbits it was largely excreted unchanged although some 12% was acetylated. It formed no glucuronide. We were unable to detect any hydroxysulphadiazine in sulphadiazine urine. The only compound isolated was N^4-acetylsulphadiazine (m.p. and mixed m.p. 245°), which was obtained in excellent yields by continuous ether extraction of sulphadiazine urine brought to pH 1 with HCl. N^4-Acetylsulphamerazine (m.p. and mixed m.p. 242°) and N^4-acetylsulphamezathine (m.p. and mixed m.p. 241°) were isolated from the corresponding urines in a similar manner.



VII

Sulphapyrazine (VII) undergoes no oxidation in the rabbit, although it is highly acetylated (88%). Biological oxidation in the pyrazine ring is unlikely on theoretical grounds, for positions 3, 5 and 6 are all deactivated by the ring nitrogens (cf. Krems & Spoerri, 1947).

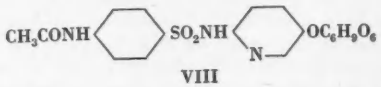
Synthesis of 2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid. The acetyl derivative of this acid was prepared according to Rose & Swain (1945). The acetyl compound (4·5 g.) was boiled for 1 hr. with 50 ml. 5N-KOH. The mixture was treated with charcoal and filtered. The hot solution was treated with dilute H_2SO_4 till strongly acid to litmus and the *carboxylic acid* separated as a yellow precipitate. After cooling to 0° the crystalline solid was

filtered off. It was sparingly soluble or insoluble in water and the usual organic solvents, and was purified by dissolving in hot alkali and precipitating with H_2SO_4. It formed pale-yellow short needles, m.p. 253–256°, and titrated as a dibasic acid. (Found: N, 18·3; equiv. by titration, 157. $C_{12}H_{12}O_4N_4S$ requires N, 18·2%; equiv. 154.) (Ultraviolet absorption spectrum: one band at $\lambda_{max.}$ 255 mμ. with $\epsilon_{max.}$ 19,400.) It forms an insoluble copper salt. This salt was prepared by dissolving the acid in dilute NaOH and making just acid with dilute HCl to give a supersaturated solution, and then adding $CuSO_4$ solution. The salt was precipitated as a pale-blue micro-crystalline powder insoluble in water, dilute HCl and acetic acid, but dissolving in cold KOH and conc. HCl. No precipitates were obtained on adding soluble Zn, Ba, Ca, Hg, Pb and Fe^{+++} salts to a supersaturated solution of the acid. The Ag salt was insoluble in water.

Sulphathiazole, sulphapyridine and sulphaguanidine

The results for these compounds and their N^4-acetyl derivatives are given in Table 3.

occurring in rabbit urine is likely to be partly acetylated. Considerations of the electron distribution on the pyridine ring suggest that the β-positions are the most likely to be oxidized, and therefore a glucuronide of sulphapyridine can be tentatively formulated as 2-(*p*-acetylsulphanilamido)-3-or-5-glucuronosidopyridine (VIII is the 5-glucuronide).



VIII

In the case of sulphaguanidine (m.p. 186°) and its acetyl derivative (m.p. 260°) absorption is so slow that glucuronide formation, if it occurred, could not be detected. Sulphaguanidine itself is highly acetylated and its acetyl derivative appears to undergo a slight deacetylation, possibly by the organisms of the gut.

Table 3. *The oxidation and acetylation of sulphathiazole, sulphapyridine and sulphaguanidine in the rabbit*

Compound fed	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated (% of dose)	Acetylated (% of total)	Oxidized as glucuronide (% of dose)
Free drugs:						
Sulphathiazole	0·3	51	30	21	41	3
	0·45	82	31	51	62	8
	1·0	92	33	59	64	6
Sulphapyridine	0·75	67	19	48	72	29
Sulphaguanidine	0·2	60	7	53	88	?*
Acetyl derivatives:						
Acetylsulphathiazole	1·0	8	0·5	7·5	94	0
Acetylsulphapyridine	0·75	62	1	61	98	28
Acetylsulphaguanidine	0·2	63	6	56	89	?*

* These drugs are too slowly absorbed to allow glucuronic acid determinations of any significance.

Sulphathiazole (m.p. 195°) is less highly acetylated than the compounds of the sulphadiazine series. It does, however, undergo a small but significant oxidation of about 6% of the dose. Its acetyl compound is very poorly absorbed, and after several days only about 8% of the dose is excreted in the urine. The deacetylation of this compound is not significant.

In the case of sulphapyridine (m.p. 196°) we have an example of a sulphonamide in which oxidation and acetylation may occur in the same molecule. The figures in Table 3 indicate that this may be a case of a compound which is first acetylated and then oxidized, for the oxidation of N^4-acetylsulphapyridine (m.p. 230°) occurs to the same extent (28%) as with sulphapyridine itself (29%). Although the acetyl compound is oxidized it does not undergo significant deacetylation. Other workers (Weber *et al.* 1943; Bray *et al.* 1946) have shown that sulphapyridine is oxidized somewhere in the heterocyclic ring. The present work indicates that the glucuronide

Sulphanilic and p*-aminobenzoic acids*

The results for these compounds and their acetyl derivatives are given in Table 4. Sulphanilic acid is excreted unchanged, there being no acetylation and no oxidation. Acetylsulphanilic acid behaves similarly, there being no deacetylation and no oxidation. These results are what would be expected, for both compounds are comparatively strong acids and the body rapidly excretes strong acids unchanged (cf. Quick, 1932*b*). Hildebrandt (1906) has reported that sulphanilic acid is excreted unchanged by dogs, but Zehender (1943) reports that it is highly acetylated in guinea pigs. *p*-Aminobenzoic acid is partly excreted unchanged, partly acetylated (20%) (cf. Harrow, Mazur & Sherwin, 1933) and partly conjugated with glucuronic acid to form an ester glucuronide (15%). This glucuronide is probably largely *p*-aminobenzoylglucuronide for *p*-acetamidobenzoic acid forms a negligible amount (4%) of glucuronide and is mainly excreted unchanged.

Table 4. *The oxidation and acetylation of sulphanilic and* p*-aminobenzoic acid in the rabbit*

Compound	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated (% of dose)	Acetylated (% of total)	Oxidized (% of dose) As glucuronide	Oxidized (% of dose) Ethereal sulphate
Sulphanilic acid	0·63	97	95	0	0	0	—
p-Aminobenzoic acid	0·4	94	72	22	23	0 (15)*	0
Acetylsulphanilic acid	0·5	99	1	98	99	0	—
p-Acetamidobenzoic acid	0·4	90	0·3	90	100	0 (4)*	—
p-Acetamidophenylacetic acid	0·5	55	0·2	55	100	—	—

* As ester glucuronide (see text).

p-Aminobenzoic acid does not undergo nuclear oxidation for it does not give rise to an ethereal sulphate. It could give rise to a glycine conjugate, but we did not look for it since Quick (1932*a*) has reported that it does not form a glycine conjugate in dogs. The work of Smith, Bayliss, Orgorzalek & McClure (1946), however, suggests that *p*-aminohippuric acid may be formed in man.

p-Acetamidophenylacetic acid (m.p. 168°) was included in this series because we wished to find out whether removal of the carboxyl group from the ring would influence the *in vivo* stability of the *p*-acetamido group. Table 4 shows that this acid is not deacetylated in the rabbit.

DISCUSSION

All the compounds studied here are derivatives of aniline in which there is a para-substituted acidic group (aniline substituted with other types of groups will be the subject of another paper). It is therefore interesting to compare the acid dissociation constants of these compounds with the extent of their acetylation in the rabbit (see Table 5).

Table 5. *The relation between dissociation constant and extent of acetylation of certain* p*-substituted anilines*

(pK_a's quoted from Bell & Roblin (1942).)

Compound	pK_a	Drug excreted as acetyl compound (average figures) (%)
Sulphanilic acid	3·24	0
p-Aminobenzoic acid	4·68	23
Sulphapyrazine	6·04	87
Sulphadiazine	6·48	72
Sulphamerazine	7·06	90
Sulphathiazole	7·12	56
Sulphamezathine	7·37	85
Sulphapyridine	8·43	72
Sulphanilamide	10·43	50

It appears from this table that there is a rough relationship between pK_a and percentage acetylation. When pK_a is small, i.e. the compound is a relatively strong acid, acetylation only takes place to small extent, e.g. sulphanilic acid, $pK_a = 3{\cdot}24$ and acetylation 0%; *p*-aminobenzoic acid, $pK_a = 4{\cdot}68$ and acetylation, 23%. Compounds with pK_a's varying from about 6 to 9 are all highly acetylated. It appears probable from other work carried out in this laboratory that when pK_a is very large, i.e. the compound is not an acid or is an extremely weak acid, then the percentage acetylation again becomes very small (e.g. aniline, unpublished results). These facts suggest that there is an optimum extent of ionization for biological acetylation. If the compound is too highly ionized either as an acid or a base, there is a tendency for acetylation to be low or zero. The alternative explanation is that if a compound is strongly acidic there is a tendency for rapid excretion and there is little time for acetylation. We found, for example, that sulphanilic acid was rapidly excreted by the rabbit.

Another fact which emerges from the present results is that acetylation of the aromatic amino group is a one way reaction. None of the acetamido compounds studied here were deacetylated by the rabbit to any significant extent. Other work in this laboratory indicates that this is true also for such compounds as acetanilide, phenacetin, *p*-acetamidophenol, etc. According to Lipmann (1946) the acetylation of the aromatic amino group in sulphanilamide involves the participation of adenosine triphosphate and acetate, and since the reaction is highly exergonic, the tendency is complete acetylation. Reversal of this reaction resulting in deacetylation is therefore unlikely. However, deacetylation could be brought about by hydrolysis, which is not the reverse of acetylation, by the mechanism suggested by Lipmann. Michel, Bernheim & Bernheim (1937) and Kohl & Flynn (1940) have reported that rat liver and kidney contains an acylase which deacetylates acetylated sulphonamides and acetanilide *in vitro*. In the intact rabbit, if this enzyme system does occur, there appear to be considerable limitations to its activity.

The present work also gives us some information on the relation of oxidation to acetylation. It appears, as far as the compounds studied here are concerned, that there are two types of amino compounds involved, (1) those in which oxidation and

acetylation require separate molecules one for each process, e.g. sulphamezathine, and (2) those in which oxidation and acetylation can occur in the same molecule, e.g. sulphapyridine (cf. also β-naphthylamine, Dobriner, Hofmann & Rhoads, 1941; and 2-acetamidofluorene, Bielschowsky, 1945). Other types of aromatic compounds, such as those which are oxidized but not acetylated, are also known and will be discussed in another communication.

SUMMARY

The extent of acetylation and of oxidation in the rabbit of certain *N*-substituted heterocyclic sulphonamide drugs has been measured. Similar measurements have been made on sulphanilic acid, *p*-aminobenzoic acid and related compounds. The following conclusions were reached:

(1) All sulphonamides with pK_a's between 6 and 9 are highly acetylated; compounds with a pK_a less than 6 were not highly acetylated.

(2) *p*-Substituted acetanilides are not deacetylated by the rabbit.

(3) In some sulphonamides, e.g. sulphamerazine, sulphamezathine and sulphathiazole, oxidation occurs in the free drug but not with the acetylated drug, whereas in sulphapyridine the acetyl derivative and the free drug are oxidized.

(4) Sulphadiazine and sulphapyrazine are not oxidized.

(5) Sulphanilic acid is neither acetylated nor oxidized whereas *p*-aminobenzoic acid is slightly acetylated (*c.* 20%) but not oxidized.

(6) The results obtained have been related as far as possible to certain physicochemical concepts.

We wish to acknowledge our gratitude to May and Baker Ltd. for gifts of sulphadiazine, sulphapyrazine, sulphathiazole and sulphapyridine, to Imperial Chemical Industries (Pharmaceuticals) Ltd. for sulphamezathine and sulphaguanidine and to Sharpe and Dohme Ltd. for sulphamerazine. The expenses of this work were in part defrayed by a grant from the Medical Research Council.

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Studies in Detoxication

15. ON THE GLUCURONIDES OF STILBOESTROL, HEXOESTROL AND DIENOESTROL

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The importance of stilboestrol, hexoestrol and dienoestrol as chemotherapeutic substances of value as oestrogens and as anticancer compounds has now been established (cf. Dodds, 1945; Haddow, 1947). Their fate in the body, however, has not been fully worked out. In 1939 Stroud (1939*a*) showed that stilboestrol, on injection into rabbits, was excreted both in a free and combined state and, in 1942, Mazur & Shorr isolated stilboestrol monoglucuronide from the urine of rabbits receiving small doses of stilboestrol by injection. The glucuronide isolated corresponded to 30% of the dose. Since stilboestrol is a phenol it would be expected to form an ethereal sulphate. Teague (1945), however, has reported that it causes no significant change in the organic sulphate output of rabbits or rats. The same worker found no change in the neutral sulphur output. These experiments with intact animals therefore suggest that stilboestrol undergoes but one change in the body, namely conjugation of one of its hydroxyl groups with glucuronic acid. *In vitro* experiments carried out by Zimmerberg (1946) (cf. Zondek, Sulman & Sklow, 1943) showed that stilboestrol disappeared when added to rat-liver slices in a Ringer-bicarbonate medium at 37°. The stilboestrol was believed to be conjugated or oxidized, only one hydroxyl being attacked. Zimmerberg suggested that conjugation could take place with sulphuric or another acid, probably glucuronic acid, depending on which is available.

In the case of hexoestrol and dienoestrol Stroud (1939*a*) showed that both these compounds were excreted in a combined form by rabbits. Up to the present time no reliable data have been available on the quantitative aspects of the conjugation of these three oestrogens. In the present paper quantitative determinations of the outputs of glucuronic acid and ethereal sulphate in rabbits following the feeding of these compounds will be presented. A description is also given of the isolation and characterization of the glucuronides. Further work is in progress on these glucuronides, for stereochemical considerations suggest that the nature of the glucuronides from hexoestrol and dienoestrol may be more complicated than is apparent at first sight.

METHODS

Animals. Rabbits (giant chinchilla), kept on a constant diet of 50 g. of Lever's cubes and 100 g. of cabbage *per diem*, were used throughout these experiments. Compounds were administered in all cases in aqueous suspension by means of a stomach tube.

Glucuronic acid was determined according to the method of Hanson, Mills & Williams (1944).

Ethereal sulphate and neutral sulphur were determined gravimetrically by the Folin method (cf. Peters & Van Slyke, 1932).

Spectroscopic observations were made with a Hilger E3 quartz spectrograph.

RESULTS

Experiments with stilboestrol

Quantitative results. These are given in Table 1, and a typical experiment is illustrated in Fig. 1. The results show that at a dose level of 150 mg./kg. an average of 71% of the dose of stilboestrol can be accounted for as a monoglucuronide excreted in the urine. The glucuronic acid output is raised above normal for about 4 days, maximum excretion of the glucuronide occurring on the second day after dosing. Neither the ethereal sulphate nor the neutral sulphur output appears to be disturbed.

Isolation of stilboestrol mono-β-D-glucuronide. Stilboestrol (0·8 g.) (British Drug Houses Ltd., m.p. 173°) was fed to each of 6 rabbits and the urines were collected each day during the next 4 days. Mazur & Shorr (1942) have reported that the sodium salt of stilboestrol glucuronide is relatively insoluble in water. Since rabbit urine is normally alkaline (pH > 8), it is reasonable to expect that the urinary deposit would contain appreciable amounts of this sodium salt. The urines were therefore centrifuged and the deposits obtained worked up separately. The deposits were triturated with small amounts of dilute H_2SO_4, and then anhydrous Na_2SO_4 was added to give a thick pasty mass. The latter was now triturated with ether (5×100 ml. portions). The ether extracts were reduced to 100 ml. and the insoluble sodium salt was precipitated on adding 30 ml. saturated $NaHCO_3$ solution. After keeping at 0° for 1–2 hr. the salt was filtered and dried *in vacuo*. The centrifuged urines were acidified with dilute H_2SO_4 and extracted with 4 or 5 portions of ether. The ethereal extracts were then treated as in the case of the deposits. The yields in a good

Table 1. *Excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving stilboestrol orally*

Rabbit no.	Wt. (kg.)	Dose (mg./kg.)	'Extra' glucuronic acid* (% of dose)	'Extra' ethereal sulphate (% of dose)	'Extra' neutral sulphur (% of dose)
81	2·6	150	77·3	—	—
87	2·85	150	73·9	—	—
89	2·75	150	67·1	—	—
90	2·9	150	66·2	—	—
97	2·35	250	—	0	—
69	2·45	250	—	0	0
82	3·0	100	—	0	—
86	2·75	100	—	0	0
80	3·1	250	—	—	0

* Calculated as a monoglucuronide.

experiment are given in Table 2. For analysis the salt (plates) was purified by recrystallization from methanol. (Found: Na, 4·7%; calc. for $C_{24}H_{27}O_8Na$, Na, 4·7%.) To obtain the free glucuronide, the sodium salt was triturated

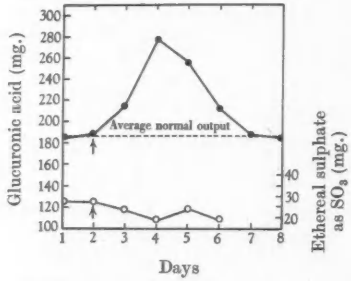


Fig. 1. The glucuronic acid and ethereal sulphate output in rabbits receiving stilboestrol orally. ●—●, glucuronic acid curve for rabbit no. 90 that received 440 mg. of stilboestrol on day 2. o—o, ethereal sulphate output in rabbit no. 86 that received 275 mg. stilboestrol on day 2.

Table 2. *Yields of sodium stilboestrol monoglucuronide from rabbit urine*

(Amount of stilboestrol fed was 4·8 g. (6 rabbits). Total Na salt recovered was 5·37 g.)

	Sodium salt from	
Day	Centrifuged urine (g.)	Urinary deposit (g.)
1	0·48	0·65
2	0·70	1·88
3	0·40	0·61
4	0·25	0·40
Totals	1·83	3·54

with dilute H_2SO_4, and then anhydrous Na_2SO_4 added to form a thick pasty mass. The glucuronide was extracted from this by stirring with several portions of ether. The ethereal extracts were taken to dryness and the residue dissolved in a small amount of acetone, from which stilboestrol monoglucuronide *dihydrate* crystallizes on adding benzene in excess. On recrystallization from benzene-acetone mixtures it forms masses of long needles, m.p. 179° (decomp.), sintering at 65–75° and showing $[\alpha]_D^{20°} = -56·5°$ ($c = 1$ in ethanol). (Found: C, 59·8; H, 6·8%; equiv. by titration, 489; glucuronic acid, 39%. $C_{24}H_{28}O_8.2H_2O$ requires C, 60·0; H, 6·7%; equiv. 481; glucuronic acid, 40·3%.) The determination of water of crystallization was not satisfactory since the compound decomposed on prolonged heating at 110°. It was sparingly soluble in cold water and benzene, but readily soluble in ethanol, acetone, ether and hot water. It gave an intense naphthoresorcinol reaction and a deep cherry-red colour in the Dingemanse $SbCl_5$ reaction (see Dingemanse, 1940).

Benzylamine salt of stilboestrol glucuronide. Pure stilboestrol-glucuronide (200 mg.) was dissolved in 20 ml. ethanol followed by 20 ml. ethyl acetate. Benzylamine (10 drops) was then added to the mixture with stirring. After a few seconds the *salt* crystallized out. After 15 min. at 0°, the mixture was filtered, and the salt washed with ethanol and dried in the desiccator (yield 210 mg., m.p. 218° decomp.). On recrystallization from 10% ethanol, the salt formed long white needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{20°} = -55·3°$ ($c = 0·172$ in 50% aqueous acetone). (Found: C, 63·75; H, 6·9; N, 2·55%. $C_{31}H_{37}O_8N.2H_2O$ requires C, 63·4; H, 7·0; N, 2·4%.) The salt was sparingly soluble in all the usual solvents.

Detection of free stilboestrol. (*a*) *In urine.* Urine from rabbits receiving stilboestrol orally was centrifuged and the deposit removed. It was then acidified and extracted with ether in a continuous extractor for several hours. The glucuronide in the ether extract was removed by six treatments with saturated sodium bicarbonate solution. The ether was now extracted with 10% (w/v) Na_2CO_3 to remove free stilboestrol. Acidification of this latter extract, followed by extraction with ether, yielded a solution giving a red colour in the Dingemanse reaction thus indicating the presence of free stilboestrol. A separate experiment showed that stilboestrol glucuronide could be completely removed, as indicated by the Dingemanse reaction, from an ethereal solution by saturated $NaHCO_3$. By this method small amounts of free stilboestrol were found in the urine for 4 days after dosing.

(*b*) *In faeces.* By a similar method free stilboestrol in small amounts was detected in the faeces of rabbits on all 4 days after dosing.

Experiments with hexoestrol

Quantitative results. These are given in Table 3, and Fig. 2 illustrates a typical experiment. These figures indicate that about 71% of hexoestrol is excreted as a glucuronide, the excretion persisting for 4–5 days after dosing. The ethereal sulphate and neutral sulphur outputs are not raised.

Table 3. *The excretion of glucuronic acid, ethereal sulphate and neutral sulphur by rabbits receiving hexoestrol orally*

Rabbit no.	Dose (mg./kg.)	Extra glucuronic acid (% of dose)	Extra ethereal sulphate (% of dose)	Extra neutral S (% of dose)
85	200	77·1	—	—
87	200	73·1	—	—
97	200	63·4	—	—
89	250	—	0	0
90	250	—	0	0

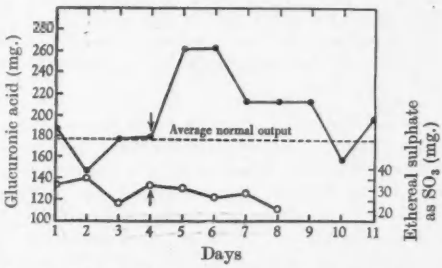


Fig. 2. The glucuronic acid and ethereal sulphate output in rabbits receiving hexoestrol orally. •—•, glucuronic acid curve for rabbit no. 85 that received 558 mg. of hexoestrol on day 4. o—o, ethereal sulphate curve for rabbit no. 90 that received 770 mg. of hexoestrol on day 4.

Isolation of p-*β*-D-*glucuronosidodiphenyl.* Preliminary experiments on the isolation of hexoestrol glucuronide indicated that its properties were not similar to those of stilboestrol glucuronide. In order to avoid undue wastage of hexoestrol, model experiments were first carried out on *p*-hydroxydiphenyl since it was thought that its glucuronide might be similar to that of saturated hexoestrol.

p-Hydroxydiphenyl (1 g.) (m.p. 164–165°; British Drug Houses Ltd.) suspended in water was fed to a rabbit by stomach tube. The urine (150 ml.) was collected after 24 hr. It gave a strong naphthoresorcinol reaction. On acidifying with HCl a fine white precipitate separated which could not be easily filtered. Extraction of the urine with ether yielded no glucuronide. The urine was therefore extracted three times in a separating funnel with a 1 : 3 ethanol-ether mixture. The extract was evaporated and a crystalline residue (0·75 g.) was left. In a similar manner, the urine for the second day yielded 155 mg. of the material. The whole was recrystallized from a large volume of hot water and p-*β*-D-*glucuronosidodiphenyl* (*p*-phenylphenylglucuronide) crystallized as needles, m.p. 189°, showing $[\alpha]_D^{20°} = -90·6°$ ($c = 1$ in 0·1 N-NaOH). (Found: C, 58·1; H, 5·6%. $C_{18}H_{18}O_7.1\frac{1}{2}H_2O$ requires C, 57·9; H, 5·7%.) It gives a strong naphthoresorcinol reaction, but only after heating with HCl and naphthoresorcinol for 2 min., i.e. twice the usual heating time (cf. dienoestrol glucuronide).

Benzylamine salt of p-*glucuronosidodiphenyl.* Ten drops of benzylamine were added gradually with stirring to 100 mg. of glucuronosidodiphenyl dissolved in 30 ml. of an equal mixture of ethanol and ethyl acetate. After 2 hr. at 0° the separated *benzylamine salt* was filtered and washed with a little ethanol (yield, 100 mg.). On crystallization from dilute aqueous ethanol, it formed colourless needles, m.p. 205° (decomp.) and showed $[\alpha]_D^{20°} = -69·7°$ ($c = 1$ in 0·1 N-NaOH). (Found: C, 65·5; H, 6·15; N, 2·9%. $C_{25}H_{27}O_7N.\frac{1}{2}H_2O$ requires C, 64·9; H, 6·2; N, 3·0%.) This salt is more soluble in cold water than the corresponding salts of the oestrogens.

Hexoestrol monoglucuronide. Hexoestrol (1 g.) (*meso* - 4 : 4′ - dihydroxy - *γδ* - diphenyl hexane, m.p. 184°; British Drug Houses Ltd.) was fed by stomach tube to each of five rabbits. The urine was collected daily for 4 days, each day's urine being worked up separately. Extraction with ether yielded very little material and the urine gave a strong naphthoresorcinol reaction only after prolonged boiling. Each day's urine was worked up as follows.

The urine (600–800 ml.) was acidified with 10 N-H_2SO_4 and then shaken with 200 ml. of a 1 : 5 ethanol-ether mixture. A copious white precipitate of inorganic material separated and this was filtered off at the pump. The filtrate was saturated with $(NH_4)_2SO_4$ thus causing an effective separation of the ethanol-ether layer which was removed. The urine layer was extracted with two further 200 ml. portions of ethanol-ether. The combined extracts (dried by anhydrous Na_2SO_4) were taken to dryness *in vacuo* at 60°. A clear brown oil remained (which crystallized in some experiments). This was boiled with 30 ml. water and, after adding charcoal, filtered. On cooling, the glucuronide separated as an oil. The oil was redissolved in the water which was then saturated with $(NH_4)_2SO_4$ and the glucuronide extracted with 1 : 5 ethanol-ether. Removal of the solvents left a partially crystalline mass which further crystallized on triturating with ether. The crystals were filtered off and the mother liquor evaporated to a gum which was used for making the benzylamine salt.

The total yield of crystalline material was 0·5 g. On recrystallization from water or benzene-acetone, *hexoestrol mono-β-D-glucuronide trihydrate* was obtained as colourless needles, m.p. 183–184° (decomp.) after sintering at 100° and showing $[\alpha]_D^{20°} - 66·3°$* ($c = 2$ in 0·1 N-NaOH). (Found: C, 57·8;

* This rotation is quoted tentatively, since we suspect resolution during recrystallization (see discussion).

H, 6·95%; glucuronic acid, 39·3%. $C_{24}H_{30}O_8 . 3H_2O$ requires C, 57·6; H, 7·25%; glucuronic acid, 38·8%.) The pure compound was insoluble in ether, benzene, light petroleum and chloroform and sparingly soluble (< 1%) in ethanol, acetone and cold water. It gives a strong naphthoresorcinol test on prolonged boiling. Since it is insoluble in chloroform, the Dingemanse $SbCl_5$ test cannot be carried out in the ordinary way. On suspending the glucuronide in dry chloroform and then warming the solution with an excess of 25% $SbCl_5$ in chloroform a pale carmine colour is produced similar to that obtained with hexoestrol itself.

The benzylamine salt of hexoestrol monoglucuronide. Some of the residual gum obtained above after separation of crystalline hexoestrol glucuronide was dissolved in a mixture of ethanol and ethyl acetate (1:1). A slight excess of benzylamine was added drop by drop with stirring. No crystals separated. The mixture was therefore warmed on a water bath and on cooling and stirring, a crystalline solid separated. The crystals (m.p. 218°) were filtered and washed with absolute ethanol and dried in a desiccator. The *salt* on recrystallization from dilute aqueous ethanol formed colourless needles, m.p. 222°, and showed $[\alpha]_D^{20°} = -48·7°$ ($c = 0·5$ in 2N-HCl in 50% aqueous ethanol). (Found: C, 62·8; H, 7·0; N, 2·3%. $C_{31}H_{39}O_8N . 2H_2O$ requires C, 63·1; H, 7·35; N, 2·4%.) It gave a strong naphthoresorcinol reaction on prolonged boiling, and a pale carmine colour in the Dingemanse reaction carried out as for hexoestrol glucuronide. It was insoluble in ethanol, ethyl acetate and cold water, but soluble in hot water.

Table 4. *The glucuronic acid output in rabbits receiving dienoestrol orally*

Rabbit no.	Dose (mg./kg.)	Glucuronic acid output: Average normal (mg./day)	Glucuronic acid output: Average output/day for 3 days after dosing (mg./day)	Glucuronic acid output: Increase (mg.)
81	200	127	132	15
82	200	154	150	—
83	200	135	146	33
84	200	160	224	192

Table 5. *Ethereal sulphate and neutral sulphur output in rabbits receiving dienoestrol orally*

Rabbit no.	Dose (mg./kg.)	Ethereal sulphate increase	Neutral S increase
98	250	0	—
68	250	0	—
89	250	0	0
90	250	0	0
87	250	0	0
81	250	0	0

Experiments with dienoestrol

Quantitative results. These are given in Tables 4 and 5 and single experiments are illustrated in Fig. 3. These tables show that dienoestrol does not disturb the ethereal sulphate and neutral sulphur output. In the case of glucuronic acid, Table 4 shows that in three cases there is apparently no disturbance of the normal glucuronic acid output. In the fourth case there is an appreciable rise corresponding to nearly 50% of the dose. Qualitative experiments (see below) have shown that a glucuronide is actually excreted and the negative results in most of our quantitative glucuronic acid estimations

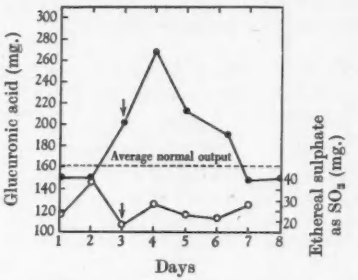


Fig. 3. The glucuronic acid and ethereal sulphate output in rabbits receiving dienoestrol orally. •—•, glucuronic acid curve for rabbit no. 84 that received 556 mg. dienoestrol on day 3. o—o, ethereal sulphate curve for rabbit no. 87 that received 930 mg. of dienoestrol on day 3.

can be ascribed to the stability of the glucuronide to acid hydrolysis. Stable glucuronides which cannot be quantitatively estimated by the naphthoresorcinol method have been described on previous occasions, e.g. euxanthic acid, *o*-aminophenylglucuronide and *p*-sulphonamido-*o*-aminophenylglucuronide (Hanson *et al.* 1944). The behaviour of dienoestrol monoglucuronide in the naphthoresorcinol procedure is being further investigated.

Dienoestrol monoglucuronide. Preliminary experiments showed that a single oral dose of 1 g. of dienoestrol (m.p. 238–239°) was not readily absorbed by rabbits and much of the compound appeared unchanged in the faeces. Therefore six rabbits were each given four separate doses of 0·25 g. during 2 days (at 11 a.m. and 4 p.m. each day). The urines were worked up daily for 5 days after the first dose, by the same method as was used for hexoestrol glucuronide. From the ether-ethanol extracts dienoestrol glucuronide was obtained as an oil which partially crystallized on triturating with ether. The residual urines after ether-ethanol extractions were then subjected to prolonged extraction (30 hr.) with ether in continuous extractors. In this way a total of 3·5 g. of crystalline glucuronide was obtained and 0·32 g. of the benzylamine salt was prepared from residues (cf. hexoestrol glucuronide). On repeated recrystallization from acidulated water *dienoestrol mono-β-D-glucuronide monohydrate* separated as

colourless needles, m.p. 181–183° (decomp.) after sintering at 120–135° and showing $[\alpha]_D^{29°} - 45{\cdot}9°$ ($c = 1$ in 0·1N-NaOH). (Found: C, 62·65; H, 6·15%. $C_{24}H_{26}O_8.H_2O$ requires C, 62·6; H, 6·1%.)

The glucuronide is insoluble in ether, benzene and light petroleum, sparingly soluble in cold water, acetone and ethanol, but fairly soluble in the latter solvents when hot. A suspension of the glucuronide in chloroform gives the same magenta colour with $SbCl_5$ (Dingemanse reaction) as does the parent oestrogen. It is remarkably stable to acid hydrolysis for it can be recrystallized from acidulated water without hydrolysis. In the qualitative naphthoresorcinol test in which HCl of at least 5N strength is used, heating for 5 min. is necessary to get a positive reaction. If the naphthoresorcinol test is carried out on the glucuronide in the normal manner, i.e. 1 min. heating, the test is negative.

The benzylamine salt of dienoestrol glucuronide. This *salt* was prepared from the gummy residues after crystallization of the free glucuronide as described for hexoestrol. It was recrystallized from dilute aqueous ethanol and formed colourless needles, m.p. 223° (decomp.), and showed $[\alpha]_D^{29°} = -35{\cdot}4$ ($c = 0{\cdot}5$ in 0·1N-NaOH). (Found: C, 63·2; H, 6·7; N, 2·6%. $C_{31}H_{25}O_8N.2H_2O$ requires C, 63·6; H, 6·7; N, 2·4%.) It behaves in the same way as the parent glucuronide in the Dingemanse and naphthoresorcinol reactions.

Spectroscopic observations

Stilboestrol. A number of workers have studied the ultraviolet absorption spectrum of stilboestrol. Elvidge (1939) reported a single maximum in the absorption curve at 240 mμ. with $\epsilon_{max.}$ 15,500–15,800. In a later paper (Elvidge, 1940) $\lambda_{max.}$ is given as 239 mμ. with $\epsilon_{max.}$ 16,000. Kharasch & Kleiman (1943) reported $\lambda_{max.}$ 250 mμ. Solmssen (1943) reported a maximum at 240 mμ. with $\epsilon_{max.}$ 15,700 and an inflexion near 280 mμ., $\epsilon = 7000$. The occurrence of this inflexion was confirmed by Kaiser & Koenig (1946), but they recorded the maximum at 250 mμ.

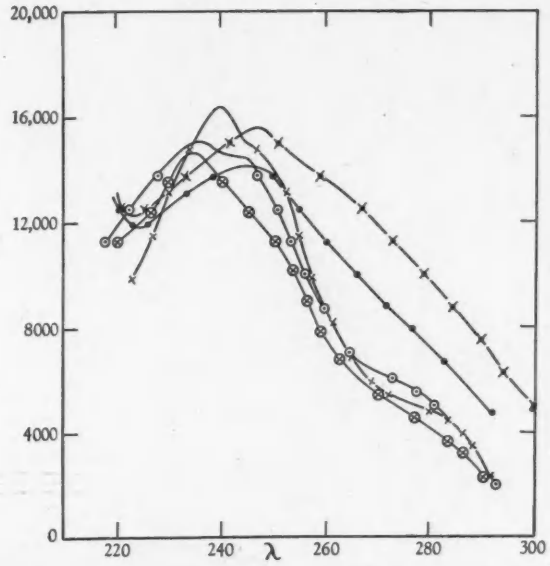


Fig. 4. Ultraviolet absorption spectra of stilboestrol and stilboestrol glucuronide. ×—× stilboestrol in absolute ethanol. ⊙—⊙ stilboestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. •—• stilboestrol in 50% (v/v) aqueous ethanol + 5 equiv. KOH. ✕—✕ stilboestrol in 50% (v/v) aqueous ethanol + excess 0·005M-KOH. ⊗—⊗ stilboestrol glucuronide in absolute ethanol.

The sample of stilboestrol used in the present work showed in ethanol solution $\lambda_{max.}$ 240 mμ., $\epsilon_{max.}$ 16,400, with inflexions near 245 mμ., $\epsilon = 15{,}000$; and 270–280 mμ., $\epsilon = 5000$ (Fig. 4).

The monoglucuronide shows $\lambda_{max.}$ 235 mμ., $\epsilon_{max.}$ 14,600 with an inflexion 270–280 mμ., $\epsilon = 5000$ (Fig. 4).

The ultraviolet absorption spectrum has also been studied in aqueous ethanol containing various

amounts of potassium hydroxide, in an attempt to obtain information regarding the ionization of the phenolic hydroxyl groups which might be correlated with the ease of biological conjugation (Fig. 4).

In 50% (v/v) aqueous ethanol containing one equivalent of KOH, the main absorption band of stilboestrol splits into two bands: $\lambda_{max.}$ 235 mμ., $\epsilon_{max.}$ 15,000; $\lambda_{max.}$ 244 mμ., $\epsilon_{max.}$ 14,500. The inflexion near 280 mμ. remains unchanged. Further addition of alkali results in the longer wave length component becoming predominant together with the disappearance of the 280 mμ. inflexion. This variation in the position of $\lambda_{max.}$ with solvent between 235 and 250 mμ. may account for the lack of agreement between different workers regarding the actual position of the band. Furthermore, if the shift towards longer wave lengths with increasing pH of the solvent is ascribed to increased ionization of the stilboestrol, then the presence of an inflexion near 245 mμ. in ethanol solution and in a solution buffered at pH 7·4 is indicative of some ionization under these conditions.

Hexoestrol. Elvidge (1939) reported two maxima in the absorption spectrum of hexoestrol $\lambda\lambda_{max.}$ 229 and 281 mμ. Kaiser & Koenig (1946) recorded $\lambda\lambda_{max.}$ 230 and 280 mμ.

It has been found, however, that the actual position of $\lambda_{max.}$ varies with the solvent. In 50% (v/v) aqueous ethanol the maxima occur at 240 and 295 mμ. with $\epsilon_{max.}$ 18,000 and 4700 respectively (Fig. 5). Addition of one equivalent of KOH to this solution shifts the spectrum to shorter wave lengths,

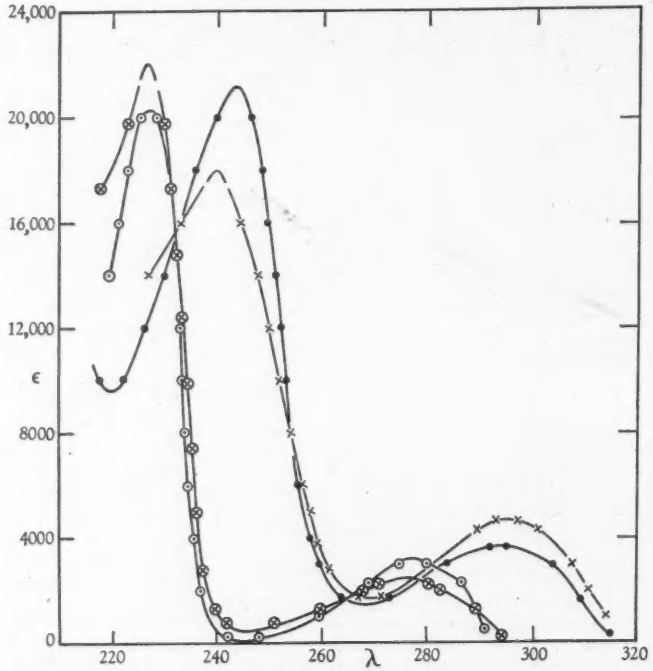


Fig. 5. Ultraviolet absorption spectra of hexoestrol and hexoestrol glucuronide. ×—× hexoestrol in 50% (v/v) aqueous ethanol. ⊙—⊙ hexoestrol in 50% (v/v) aqueous ethanol + 1 equiv. KOH. •—• hexoestrol in 50% (v/v) aqueous ethanol + excess 0·005 M-KOH. ⊗—⊗ hexoestrol glucuronide in absolute ethanol.

$\lambda\lambda_{max.}$ 227·5 and 278 mμ. with $\epsilon_{max.}$ 20,200 and 3200 respectively (Fig. 5). Further addition of alkali up to five equivalents has little effect, but in excess of alkali (0·005 M in a solution M × 10^{-4} with respect to hexoestrol) the spectrum reverts to that of hexoestrol in aqueous ethanol, showing $\lambda\lambda_{max.}$ 243·5 and 293·5 mμ. with $\epsilon_{max.}$ 21,200 and 3700 respectively (Fig. 5).

Hexoestrol glucuronide in ethanol shows $\lambda\lambda_{max.}$ 227 and 276 mμ. with $\epsilon_{max.}$ 22,000 and 2400 respectively (Fig. 5).

It is difficult to interpret these results on the basis of ionization of hexoestrol, particularly since the

shift to shorter wave lengths on addition of one equivalent of KOH is contrary to the usual behaviour of phenolic compounds. (Phenol itself shows $\lambda\lambda_{max.}$ 215 and 273 mμ. in ethanol solution and $\lambda\lambda_{max.}$ 230 and 285 mμ. in alkali.) It seems more likely that the variations are to be ascribed to a solvent effect only, the effects of any ionization changes being subsidiary.

measurements show that at least 70% of the drugs is conjugated with glucuronic acid and this is probably true also for dienoestrol. This conjugation probably results in a detoxication and an inactivation of these compounds. Not one of the three compounds appears, from quantitative data, to form a mercapturic acid or an ethereal sulphate. The absence of mercapturic acid formation is not

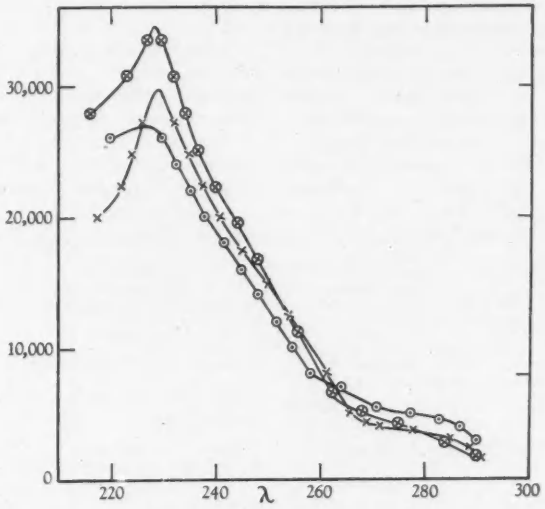


Fig. 6. Ultraviolet absorption spectra of dienoestrol and dienoestrol glucuronide. ×—× dienoestrol in absolute ethanol. ⊙—⊙ dienoestrol in 50% (v/v) aqueous ethanol+1 equiv. KOH. ⊗—⊗ dienoestrol glucuronide in absolute ethanol.

Dienoestrol. The sample of dienoestrol shows an absorption spectrum similar in general shape to that of stilboestrol, with $\lambda_{max.}$ 229 mμ., $\epsilon_{max.}$ 30,000. There is also an inflexion in the curve between 275 and 280 mμ. with $\epsilon = 4000$ (Fig. 6). Elvidge (1940) gives $\lambda_{max.}$ 228 mμ., $\epsilon_{max.}$ 26,600. There is little change in spectrum with variation in solvent pH, whilst the glucuronide shows $\lambda_{max.}$ 228 mμ., $\epsilon_{max.}$ 34,700, with an inflexion near 275 mμ., $\epsilon = 4000$ (Fig. 6).

These spectra give little information regarding any possible ionization of the dienoestrol molecule. It is probable that the absorption maximum at 229 mμ. has its origin in the ethylenic double bonds, and is little affected by ionization of the phenolic hydroxyls.

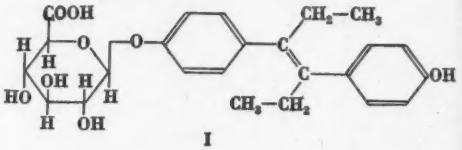
DISCUSSION

The present results indicate that in the rabbit the main metabolic change undergone by the three synthetic oestrogens studied is conjugation with glucuronic acid to form monoglucuronides. In the cases of stilboestrol and hexoestrol quantitative

surprising for all three are para-substituted compounds and therefore the position in the aromatic ring where acetylcysteine conjugation normally occurs is blocked. Furthermore there is no known case of an aromatic hydroxy compound forming a mercapturic acid. However, the apparent absence of ethereal sulphate conjugation is surprising, for all three compounds contain phenolic hydroxyl groups, the prerequisites for sulphate conjugation. There is of course the possibility that ethereal sulphates are slowly formed and excreted, but that their concentration in the urine is too low to be detected by the quantitative method used. This possibility is being investigated. If the absence of ethereal sulphates is confirmed, then we must look to the nature of the hydroxyl groups for the reason, for phenols are known which, when fed to animals, do not conjugate as ethereal sulphate, e.g. salicylic acid (Williams, 1938). In the case of salicylic acid the phenolic hydroxyl is not free as a result of hydrogen bonding. It is possible that ethereal sulphate formation is related to the ionization of phenolic hydroxyl groups

for it takes place only with phenols. Ethereal sulphate formation with other types of hydroxyl groups, such as those found in alcohols, has never been recorded. In the alcohols it is known that the OH groups are extremely feebly ionized (e.g. glycol, $K_a = 6 \times 10^{-15}$; glycerol, $K_a = 7 \times 10^{-15}$) whereas in phenols the ionization constants vary between $K_a = 5{\cdot}6 \times 10^{-8}$ for *p*-nitrophenol to about 1×10^{-10} for phenol (see Britton, 1942). This suggests a small optimum ionization of the OH group for ethereal sulphate formation. The ionization constants of the synthetic oestrogens have not been recorded. Our spectroscopic data indicate that stilboestrol will ionize in alkaline solution, but the data for hexoestrol and dienoestrol do not permit of any conclusion in this respect. The relation between ionization constants and ethereal sulphate formation is being further investigated.

That stilboestrol has a *trans*-structure has, however, been questioned on spectroscopic grounds by Solmssen (1943).



I

In the case of hexoestrol, the position is not quite so clear, since hexoestrol is an internally compensated compound, i.e. *meso*-3:4-di(*p*-hydroxyphenyl)-hexane. Conjugation of one of the hydroxyls of hexoestrol with glucuronic acid destroys this internal compensation and therefore two enantiomorphous hexoestrol monoglucuronides (II and III)

Table 6. *The properties of the monoglucuronides of synthetic oestrogens*

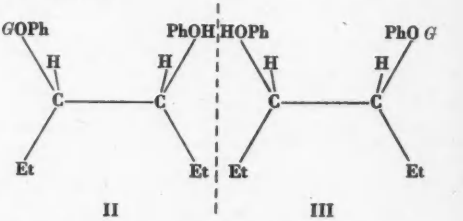
Property	Stilboestrol glucuronide	Hexoestrol glucuronide	Dienoestrol glucuronide
Hydration	$2H_2O$	$3H_2O$	$1H_2O$
M.p.	179°	183–184°	181–183°
$[\alpha]_D$	−56·5° (in ethanol)	−66·3°* (in 0·1 N-NaOH)	−45·9° (in 0·1 N-NaOH)
Ultraviolet absorption spectra in ethanol	$\lambda_{max.}$ 235 mμ. $\epsilon_{max.}$ 14,600 ~270–280 mμ. 5,000	$\lambda_{max.}$ 227 mμ. $\epsilon_{max.}$ 22,000 276 mμ. 2,400	$\lambda_{max.}$ 228 mμ. $\epsilon_{max.}$ 34,700 ~275 mμ. 4,000
Stability to acid hydrolysis	Easily hydrolyzed	Hydrolyzed with some difficulty	Hydrolyzed with much difficulty
M.p. of benzylamine salt	223°	222°	223°

* See discussion.

The nature of the glucuronides of the synthetic oestrogens also calls for some comment (see Table 6). They vary in their stability to acid hydrolysis, for whereas stilboestrol glucuronide is relatively easily hydrolyzed, dienoestrol glucuronide is hydrolyzed only with difficulty, hexoestrol glucuronide taking an intermediate position. All three are hydrated which is a common feature of many glucuronides. It has been frequently observed in this laboratory that a large number of glucuronides do not crystallize without water of crystallization and removal of this water usually results in the production of a non-crystalline glass. The negative rotations of the three glucuronides indicate that, like all biosynthetic glucuronides, they are β-glycosides.

The exact structures of these glucuronides have yet to be determined for a consideration of the stereochemistry of hexoestrol and dienoestrol indicates that the biosynthesis of more than one monoglucuronide is possible in these cases. The structure of stilboestrol glucuronide is straightforward because, if we accept a *trans*-stilbene structure for stilboestrol, then the glucuronide can be formulated as 4-(β′-D-glucuronosido-)-4′-hydroxy-α:β-diethyl-*trans*-stilbene (I).

should arise. This point is being further investigated, for it is quite possible that the glucuronide isolated is a racemate.

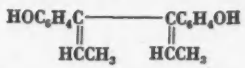


where —PhO*G* = —$C_6H_4OC_6H_9O_6$.

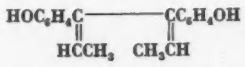
Similar difficulties arise with dienoestrol, but for slightly different reasons. As far as we are aware, the configuration of dienoestrol has not been established, but we know that it is a 3:4-di(*p*-hydroxyphenyl)-hexa-2:4-diene.

If it has structure (IV), then two monoglucuronides are possible depending upon which hydroxyl is conjugated. With structures (V) and (VI) only one monoglucuronide is possible in each case. These points are being further investigated.

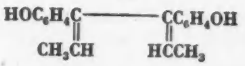
Stroud (1939*b*) has shown that stilbene when injected into rabbits gives rise to hippuric acid, which indicated splitting and oxidation at the double bond.



IV



V



VI

A similar change is therefore possible with stilboestrol, the product in this case being *p*-hydroxybenzoic acid. We have examined stilboestrol urine for *p*-hydroxybenzoic acid, but were unable to find it. We conclude therefore that, in the rabbit, the double bond in stilboestrol is resistant to biological attack.

SUMMARY

1. A preliminary study has been made of the fate of stilboestrol, hexoestrol and dienoestrol in the rabbit.

2. The preparation and properties of the monoglucuronides of stilboestrol, hexoestrol and dienoestrol prepared by feeding these oestrogens to rabbits are described.

3. The benzylamine salts of the above glucuronides have been prepared and are described.

4. The isolation of *p*-glucuronosidodiphenyl from the urine of a rabbit receiving *p*-hydroxydiphenyl is described, together with its benzylamine salt.

5. Some 70% of stilboestrol and of hexoestrol is excreted by rabbits combined with glucuronic acid. In the case of dienoestrol the amount conjugated is uncertain because dienoestrol monoglucuronide is highly resistant to acid hydrolysis and hence its determination by the method of Hanson, Mills & Williams (1944) is uncertain.

6. None of the three oestrogens affects the ethereal sulphate and neutral sulphur level of rabbit urine. These results are discussed and the possible reasons for them indicated.

7. The absorption spectra of the three oestrogens and their glucuronides under various conditions have been studied.

8. There is no indication that stilboestrol is degraded *in vivo* to single ring compounds.

9. The stereochemistry of hexoestrol and dienoestrol glucuronides is discussed.

The work was carried out at the request of the Oestrogens in Cancer Committee (Chemical Panel) of the Royal Society of Medicine. The synthetic oestrogens used in this work were given by the British Empire Cancer Campaign. One of us (A.L.S.) participated in this work as a holder of an I.C.I. Fellowship.

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The Estimation of a Fraction Resembling Pregnanediol Glucuronide in Human Urine

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The amount of pregnanediol in human urine can be estimated by an assay of the (partially purified) sodium pregnanediol glucuronidate (Na*PG*) extracted by butanol (Venning, 1937, 1938), or of the pregnanediol itself, extracted from the urine after hydrolytic treatment (Astwood & Jones, 1941; Talbot, Berman, MacLachlan & Wolfe, 1941). The latter method is capable of considerable specificity and sensitivity and has been extensively developed. Jayle, Crépy & Wolf (1943) and Jayle & Libert (1946) used methods in which the glucuronidate, purified after isolation from butanol extracts, was estimated by the naphthoresorcinol reaction, and Allen & Viergiver (1941) precipitated the glucuronidate from butanol extracts with lead nitrate and estimated reducing substances after acid hydrolysis.

The present work arose out of an attempt to improve existing methods, and it was decided in the first place to employ the naphthoresorcinol reaction for estimating glucuronide. According to Bucher & Geschickter (1941), free pregnanediol apparently does not occur in human urine. It seemed to us that the method of Jayle *et al.* (1943) incorporated many of the known sources of error of the original Venning method, which is admittedly not suitable for very small amounts of Na*PG*, whilst that of Allen & Viergiver (1941) likewise seemed better adapted for assay of quantities larger than those which we wished to investigate. We have, therefore, devised a technique in which the glucuronidate is 'entrained' (presumably as the barium salt, cf. Westphal, 1944) on a precipitate of barium phosphate, and is estimated after isolation in this way. This method enabled us to obtain accurate recoveries of small amounts of Na*PG* after butanol extraction from pure solutions. It was found, however, that when applied to urine of normal men, or of women in the follicular phase of the menstrual cycle, it indicated the presence of glucuronidate, resembling Na*PG* closely in its solubility in butanol and its behaviour on entrainment, in great excess of the amount of Na*PG* which would be expected. The total glucuronide found in this fraction we have called 'pregnanediol glucuronide-like glucuronide', or *pregnanediol-like glucuronide* (*PLG*) (Bisset, Brooksbank & Haslewood, 1947) and we have attempted to estimate it. We have introduced a modification into the naphthoresorcinol method.

METHODS

(Including results of standardization of the method of estimating Na*PG*)

Abbreviations. Na*PG*(V) = a crystalline specimen of glucuronidate, prepared by the method of Venning (1937, 1938). Na*PG*(M) = purified glucuronidate, freed from ketonic material (Sutherland & Marrian, 1947). We found 1 mg. of Na*PG*(V) ≡ 0·859 mg. Na*PG*(M) ≡ 0·777 mg. of anhydrous sodium pregnanediol glucuronidate (Na*PG*) (Marrian & Gough, 1946). 1 mg. Na*PG* ≡ 0·618 mg. of pregnanediol. Na*PG*(V) was kept in a vacuum desiccator over H_2SO_4 until weighed out for use. Analysis indicated that, after this treatment, Na*PG*(V) contained about 1·5 mol. H_2O. Solutions of sodium pregnanediol glucuronidate were kept at 0–5°.

Purification of butanol. *n*-Butanol (Commercial Solvents Ltd.) was distilled at 112–118° over KOH (5 g./l.). It was then redistilled at *c.* 70°/20 mm., with an air leak and in the presence of 1 g./l. of 4-aminophenol and 1 ml./l. of 5 N-NaOH. It was finally redistilled at *c.* 60–70°/20 mm. This process removed substances sometimes present which tended to destroy Na*PG* when solutions with butanol were evaporated *in vacuo*.

Test of purified butanol. A volume of 250 ml., with 2 or 3 ml. of Na*PG*(V) solution (50 mg./100 ml.), was completely evaporated *in vacuo* as described below for urine extracts. The residue was heated to *c.* 90° with 10 ml. of water, the cooled solution was filtered and the glucuronidate in 2 ml. of the filtrate (≡ 0·2 mg. or 0·3 mg. Na*PG*(V)) was entrained and estimated as described under 'entrainment of glucuronidate' (below). Allowing for known losses, a recovery of not less than 95% was considered satisfactory.

Naphthoresorcinol solution. A small excess over the amount of naphthoresorcinol (Hopkin and Williams) needed for the day's experiments was weighed out and dissolved at *c.* 50° in water to make a 0·025 M-solution, which was then filtered. In earlier experiments, naphthoresorcinol solution (0·025 M) was made and 'aged' as described, e.g., by Maughan, Evelyn & Browne (1938) and Hanson, Mills & Williams (1944): this procedure is discussed below.

The naphthoresorcinol reaction

It is clear from the literature that there is little agreement as to the best method of preparing the naphthoresorcinol reagent. We have found that the amount of final extractable colour produced with a given quantity of Na*PG* varies with the age of the naphthoresorcinol solution used, reaching a peak after about 2 days' storage at 0–5°, and then

tending to become less: the reagent 'blank' increases (cf. Maughan *et al.* 1938). With old naphthoresorcinol solutions the final colour tends to be obviously more red. We have found also that fresh naphthoresorcinol solution may give very inconstant amounts of colour with the same amount of Na*PG*. This state of affairs militates seriously against the accuracy and reproducibility of the method. The facts suggest that the actual formation of coloured matter in the reaction is not in fact due to interaction of naphthoresorcinol and glucuronic acid, but rather that the effective reagent is some unstable oxidation product of naphthoresorcinol; this product, we found, is not, as appeared possible, 3-hydroxy-1:4-naphthoquinone. We therefore considered the possibility of adding to fresh naphthoresorcinol solution a weak oxidizing agent and thus effecting at once a reproducible 'ageing' process. Dr R. V. G. Ewens suggested, *inter alia*, $K_3Fe(CN)_6$ for this purpose and we found it satisfactory. One ml. of 0·05 M-$K_3Fe(CN)_6 \equiv 2$ ml. of naphthoresorcinol solution (0·025 M). We therefore first tried the effect of adding 0·1, 0·2, 0·4 . . . 1·0 ml. of 0·05 M-$K_3Fe(CN)_6$ to the reaction tubes. With 0·6–1·0 ml. $K_3Fe(CN)_6$ the final colours were increasingly red and the reagent 'blank' much increased. The use of 0·1 ml. 0·05 M-$K_3Fe(CN)_6$, however, gave an appearance during the reaction very similar to that given by 'aged' naphthoresorcinol solutions, a low 'blank' and constancy of colour. We therefore used this amount, and employed fresh, filtered naphthoresorcinol solution, as described above.

Estimation of Na*PG*. Quantities were measured into wide test tubes (15 × 2 cm.) as follows:

Test	Blank
Na*PG* solution + water to make 1·4 ml.	1·4 ml. water
0·1 ml. 0·05 M-$K_3Fe(CN)_6$	0·1 ml. 0·05 M-$K_3Fe(CN)_6$
* 2·0 ml. 2 N-HCl + 1·5 ml. 9 N-HCl	* 2·0 ml. 2 N-HCl + 1·5 ml. 9 N-HCl
2·0 ml. fresh naphthoresorcinol solution (see above)	2·0 ml. fresh naphthoresorcinol solution

The contents of the tubes were then thoroughly mixed and the colour developed, extracted and estimated as described below for urine extracts.

Reproducibility. With the above method, a series of eight results with 0·15 mg. of Na*PG*(V) gave (colorimetric readings; Ilford 404 green filter): average *E*, 0·3254, σ of an individual reading, ±0·0036 ('blank', 0·03). Eight results with 0·1 mg. of Na*PG*(V) as 'standard' at different times gave (difference between 'test' and 'blank' colorimeter readings; Ilford spectrum yellow filter): average *E*, 0·2795, σ of an individual reading, ±0·0076.

Proportionality. Beer's law was fairly closely obeyed between 0·05 and 0·25 mg. Na*PG*(V) (0·04–0·20 mg. Na*PG*). Smaller amounts were not examined, and above 0·2 mg. Na*PG*, the colour increased only slightly. Fig. 1 shows results of a proportionality experiment.

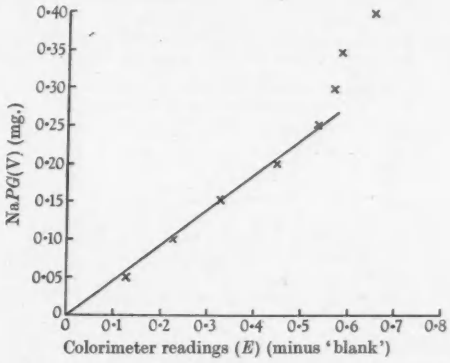

Fig. 1. Relationship between colorimeter readings and Na*PG*(V) estimated. Ilford 404 green filter.

The slight deviation from proportionality at the lower values we believe to be due largely to the impossibility of controlling the heating conditions (cf. Mills, 1946). According to Hanson *et al.* (1944) hydrolysis and colour development (in approx. 4·5 N-HCl) may not be complete for 4 hr. and we do not believe that the reaction in our conditions (2·5 N-HCl) is complete in 2·5 hr. However, the use of the reduced time does not appear to introduce a serious error, except perhaps when an artificial standard is used (see below). Solutions found to give colorimetric readings above the figure for 0·2 mg. Na*PG* (0·25 mg. Na*PG*(V)) were suitably diluted and re-estimated.

Entrainment of glucuronidate. In preliminary experiments we found that pregnanediol glucuronidate could be entrained on precipitates of $BaSO_4$ or of barium phosphate and, to a lesser extent, on other precipitates. Entrainment did not occur effectively at concentrations of sodium pregnanediol glucuronidate below about 5 mg./100 ml. and the process was, therefore, not directly applicable to urine; however, we decided to apply it to urine concentrates made with butanol. Barium phosphate was used as the entraining precipitate because it could be dissolved in the HCl used for the naphthoresorcinol reaction. Entrainment from 2 ml. of aqueous solutions of Na*PG* diluted with 2 ml. of water was carried out exactly as described for urine extracts (below): the precipitates were not washed.

Losses on entrainment. These are shown in Table 1. The results are possibly not inconsistent with the assumption that there is a loss (perhaps due to solubility) of an almost constant amount of glucuronidate on entrainment. A figure which we used in later experiments (see below) was 0·05 mg. of Na*PG*(V).

Washing of precipitates. To test the washing procedure used for precipitates from urine extracts, glucuronidate was entrained on barium phosphate as described for urine (below) and the precipitates, drained from supernatant liquid, were washed as described for those from urine extracts. Losses on washing by this method are shown in Table 2.

* 3·5 ml. of 5 N-HCl could be used: we used acid of the two strengths because we had to do so in entrainment experiments and wished to test the procedure.

Table 1. *Losses on entrainment of sodium pregnanediol glucuronidate*

(Results expressed as pure Na*PG*.)

Na*PG* entrained (mg.)	Av. loss (%)	σ of an individual % loss	No. of determinations
(*a*) Losses on entrainment from 2 ml. of Na*PG*(V) solution diluted with 2 ml. of water			
0·39	10·3	±4·4	6
0·31	15·9	±3·2	6
0·23	14·3	±3·4	6
0·16	20·6	±4·2	5
0·08	37·2	±8·3	6
(*b*) Losses on entrainment from 2 ml. of Na*PG*(M) solution diluted with 2 ml. of water			
0·36	11·2	±3·5	6
0·18	13·9	±2·3	6

Table 2. *Losses on washing entrained glucuronidate (from* Na*PG*(V))

Amount entrained (as mg. Na*PG*)	Av. % lost on washing	σ of an individual % loss	No. of determinations
0·31	1·0	±0·45	12
0·23	1·3	±0·73	12
0·16	4·5	±0·73	12
0·08	8·0	±1·94	12

The actual losses varied between 0·003 and 0·007 mg., as Na*PG*.

Extraction of Na*PG*(V) *with butanol.* Portions of 4 ml. each of a solution of Na*PG*(V) (50 mg./100 ml.) were added to 250 ml. of water containing 5 g. zinc acetate. The mixture was extracted with butanol, and the butanol was washed and evaporated to dryness as described for urine extracts (below).

The residue was heated almost to boiling with 10 ml. of water, and the cooled extract filtered; 2 ml. of the filtrate were diluted to 4 ml. and the estimation of glucuronidate (entrained but not washed) carried out as described for urine extracts (below). In two experiments losses were as follows (entrainment losses subtracted): Na*PG*(V) (as mg. Na*PG*) added to 250 ml. water, 1·55, 1·55; extraction loss, 15·5, 17·5%.

Preservation of urine. We found this most important in the estimation of *PLG* output (see, especially, Bucher & Geschickter, 1941). For example, in one series of 24 hr. collections, unpreserved urine, kept cold, was found to contain no measurable *PLG*, although the final (night) specimen had been added not more than 6 hr. before analysis. After experimenting, we finally added 3 g. of a 2:1 (w/w) mixture of finely powdered thymol and salicylic acid to the Winchester bottle in which absolutely fresh urine specimens were collected. Such urine (10 samples) showed no visible growth of micro-organisms after 48 hr. at 37°. The *PLG* content of preserved urine, kept at 37°, from a subject in the 9th month of pregnancy, did not fall until after 48 hr. As a routine, we kept urines, preserved as stated, at 0–5° prior to the analysis, which was in any case carried out as soon as was practicable.

Preliminary treatment of urines. To 300 ml. of urine we added 6 g. of zinc acetate. The mixture was shaken and filtered. This process made subsequent butanol extraction easier, and did not appear to affect the results of *PLG* determinations on urine from non-pregnant women. Without exhaustive experiments, one might hesitate to apply it to urines of very high *PLG* contents because of possible losses in the precipitate produced with zinc acetate: in a few experiments we have not observed such losses.

Estimation of PLG in urine. Urine (300 ml.), preserved as above, was treated with zinc acetate (6 g.), and the mixture was shaken and filtered; 250 ml. of the filtrate were mixed by rotation in a separating funnel with successive lots of 100, 50 and 50 ml. of purified butanol. Complete clearing of the lower layer was not awaited longer than *c.* 5 min. The combined butanol extracts were washed by rotating with 100 ml. of 0·2 N-NaOH and then with two separate lots of 20 ml. of NaCl solution (2%, w/v): separation of layers was rapid and apparently complete. The butanol was now evaporated at 50–55°/*c.* 20 mm. and the residue finally dried by evaporation with ethanol (dried over CaO) *in vacuo*. The residue was dissolved in one of two ways: either (*a*) it was heated almost to boiling with $(10 \times 1500/x)$ ml. of water (where x ml. was the 24 hr. volume of the urine), or 10 ml. of water where the 24 hr. volume was unknown or unimportant; or (*b*) the residue was dissolved in 2 ml. of N-NaOH in the cold, followed by 2 or 3 ml. water; phenolphthalein indicator was added, and such quantities of N and then of 0·1 N-HCl as were required to discharge the pink colour, water being finally added to make the final volume $(10 \times 1500/x)$ or 10 ml. as above.

The butanol residue from urine extracts contained a certain amount of acidic gums, as well as preservative, and it proved difficult to dissolve completely all the glucuronidate it contained unless heating or alkali were employed. It was also very important that the final aqueous extract of the residue should not be so alkaline as to produce a precipitate when $BaCl_2$ was added (see below), for such a precipitate would entrain extra, and uncontrollable, amounts of glucuronidate. The effect of making the final volume $(10 \times 1500/x)$ ml. was to 'correct' to a standard 24 hr. volume of 1500 ml. of urine, and so to obtain successive urine extracts in approximately the same concentration, whatever the actual 24 hr. volume: since *PLG* entrained depends partly on concentration (see below), we considered this manœuvre to be important. The solution of the residue, made as above, was filtered. A 2 ml. portion of the filtrate (*F*), in a 15 ml. conical centrifuge tube, was mixed with 2 ml. of water. The solution was treated with 1 ml. of 0·5 M-$BaCl_2$, followed, after it had been cooled for 10 min. in ice, by 1 ml. of Na_2HPO_4 (0·65 M, containing 50 ml. of N-NaOH/litre).

The tube was stoppered, the contents well mixed and again allowed to stand for 10 min. at 0°. Acetone (0·5 ml.) was used to wash the stopper and sides of the tube, so as to form an upper layer. The tube was then centrifuged at 2300 r.p.m. (radius of centrifuge head, 16·2 cm.) for 10 min. The supernatant fluid was poured off and the tube drained by inversion on filter paper. Acetone (2 ml.) (previously distilled over CaO) was added and stirred up with the precipitate with a fine wire; the tube was centrifuged for 10 min., the supernatant fluid discarded and the precipitate drained as before. The tube was then washed out with 10 ml. of saturated barium phosphate solution (added

from a pipette *without* disturbing the precipitate), centrifuged for 5 min. and drained as before: this last washing was repeated. Finally, the precipitate was dissolved in 4 ml. of 2N-HCl. 'Blank' barium phosphate precipitates (from 4 ml. of water, then $BaCl_2$ and Na_2HPO_4 as above) were made and centrifuged: these were not washed; they were dissolved in 4 ml. of 2N-HCl.

Wide test tubes (15 × 2 cm.) were now prepared as follows:

Test (duplicated)	Standard (duplicated)	Blank
0·5, 1·0 or 2·0 ml. of HCl solution of ppt. and 2N-HCl to make 2 ml.	0·2 or 0·3 ml. Na*PG*(V) solution (50 mg./100 ml.)	—
1·4 ml. water	1·2 or 1·1 ml. water	1·4 ml. water
—	2·0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above	2·0 ml. 2N-HCl solution of 'blank' barium phosphate ppt. made as above

(All tubes) 1·5 ml. 9N-HCl, 0·1 ml. 0·05M-$K_3Fe(CN)_6$ and 2·0 ml. naphthoresorcinol reagent (above)

The contents of each tube were thoroughly mixed and small funnels were placed in the mouths of the tubes, which were suspended by rubber bands clear of the bottom of a water bath. The water was heated to 100° for 2·5 hr., the contents of each tube being again mixed shortly after heating had begun. The tubes were now cooled in ice and the contents of each in turn poured and washed into a small separating funnel with 14 ml. of ethanolic ether (20% (v/v) ethanol/peroxide-free ether). The stoppered funnel was vigorously shaken and, after separation, the aqueous portion of the contents run off. The coloured extracts were poured as quickly as possible from the mouth of the funnel into colorimeter tubes, which were immediately stoppered. The colours, which were stable for some time in absence of strong daylight (see below), were measured in a King (1946) type of single-cell photoelectric colorimeter (Gambrell Ltd.) with an Ilford 404 green or Spectrum yellow filter. The 'blank' reading was subtracted from each of the others.

Calculation. Since the standard contained 0·15 or 0·1 mg. Na*PG*(V), the concentration of *PLG* (as Na*PG*(V)) was

$$\frac{\text{reading of 'test' (minus 'blank')}}{\text{reading of standard (minus 'blank')}} \times 0{\cdot}15 \text{ or } 0{\cdot}1 \text{ mg. in } 0{\cdot}5,$$

1·0 or 2·0 ml. of HCl solution of barium phosphate precipitate. Hence the amount present in 4 ml. of HCl solution, i.e. entrained on to the precipitate from a known volume of urine, was calculated. Corrections for entrainment, washing, or butanol-extraction losses could not be applied, because of ignorance of the chemical nature and physical properties of *PLG*. The figures, as Na*PG*(V), could be corrected by reference to Na*PG*(M) and expressed as Na*PG* or pregnanediol (see above).

For urines very rich in *PLG*, e.g. those of late pregnancy, smaller amounts could be extracted and time thus saved in butanol evaporation. For some experiments, we were able to use 30 ml. of pregnancy urine, with one-tenth of the quantities of butanol, etc., for the extraction and washing process: the results agreed with those of larger-scale experiments.

With such urines, quantities of less than 2 ml. of the filtered aqueous extract (*F*) of the butanol residue could be diluted to 4 ml. with water, before entrainment, or amounts of less than 0·5 ml. of HCl solution of barium phosphate precipitate could be used for the estimation.

When larger amounts than 2 ml. of the filtrate (*F*) were used for entrainment, much higher results for *PLG* were often obtained (see under RESULTS). We do not consider that the extra glucuronidate thus entrained is all '*PLG*'.

Use of an artificial standard. In view of the difficulty of obtaining a suitable glucuronide of known purity as a standard, an artificial standard would clearly be of great value in the naphthoresorcinol reaction. Examination of the final coloured extract, prepared under our conditions, with a Hilger-Nutting spectrophotometer showed that the extract, provided it was kept in shadow, had almost constant absorption between 560 and 600 mμ., although its colour altered on exposure to strong daylight. It therefore seemed possible to use the grey solution of Thomson (1946) as a standard, with the Ilford Spectrum yellow light filter. Thomson's solution was diluted to twice its volume with water and in 17 separate experiments the ratio

$$\frac{\text{grey solution (colorimeter readings)}}{\text{0·078 mg. Na}PG\text{ (colorimeter readings } \textit{less} \text{ 'blank')}}$$

gave, average, 1·24, σ of an individual reading, ±0·06. In 16 determinations, the ratio grey solution/0·117 mg. Na*PG* was, average, 0·92, σ of an individual reading, ±0·03.

For clinical use the method could probably be shortened, with some loss of accuracy, by reducing the time of heating in the naphthoresorcinol reaction to 0·5 hr. In this case some preparation of Na*PG* would have to be used as a standard, since the factors quoted above for Thomson's grey solution are for a 2·5 hr. heating period. We have found Beer's law to be less accurately obeyed for a 0·5 hr. heating period, so that the Na*PG* standard would need to approximate to the 'test' in concentration. Collection of 24 hr. urine specimens could be avoided, in individuals whose creatinine output can be assumed constant, by expressing the *PLG* as mg./g. of creatinine excreted (Jayle & Libert, 1946).

RESULTS

Constancy of PLG estimations. The results of four sets, of three estimations each, by the above method on different (male) urines were (in mg. Na*PG*/l.):

Set 1	Set 2	Set 3	Set 4
3·50	3·12	3·73	7·59
3·23	2·56	4·99	6·55
2·86	2·84	5·26	6·62
Av. 3·20	2·84	4·66	6·92

Recovery of Na*PG*(V) *added to urine extracts.* (The results given below and later, except for the female cycle, were obtained with 'aged' naphthoresorcinol,

without $K_3Fe(CN)_6$.) Na*PG*(V) solution was added to 2 ml. of the filtered aqueous solution (*F*) of the butanol residues from urines, prepared as above. *PLG* was entrained, washed and estimated as described. Standards were entrained (not washed) amounts of 0·4 mg. of Na*PG*(V); 0·05 mg. Na*PG*(V) was assumed as entrainment loss (see above). Results per 2 ml. of filtered aqueous solution (*F*) of butanol residue, were:

Na*PG*(V) added (mg.) (0·05 mg. entrainment loss subtracted)	*PLG* estimated (as mg. Na*PG*(V))	Na*PG*(V) recovered (mg.)	Recovery (%)
0	0·350	—	—
0·150	0·489	0·139	92·6
0·350	0·730	0·380	108·4

Recovery of Na*PG*(V) *added to urine.* Na*PG*(V) in solution (50 mg./100 ml.) was added to 300 ml. of female 'follicular' urine, preserved as described. The whole process of *PLG* estimation was carried out, using standards of 0·4 mg. of entrained Na*PG*(V) as above. Results are shown in Table 3.

Table 3. *Recovery of* Na*PG*(V) *added to 'follicular' urine*

Na*PG*(V) added to 250 ml. urine (16% butanol extraction loss and 0·05 mg. entrainment loss subtracted) (mg.)	*PLG* found (as mg. Na*PG*(V))	Na*PG*(V) recovered (mg.)	Recovery (%)
0	3·06	—	—
2·20	4·54	1·48	67·2
0	1·77	—	—
2·32	3·79	2·02	87·1
0	1·85	—	—
2·25	3·78	1·93	85·8
0	2·78	—	—
0·92	3·70	0·92	100·0
0	3·33	—	—
0·84	4·37	1·04	124·0

The recovery figures given in Table 3 are insufficient to form a reliable statistical basis for calculating the error of the whole method, and, in any case, they are somewhat unreal because of the unknown (and presumably variable) properties of *PLG*. However, they serve to show that the method should allow the estimation, with a moderate degree of accuracy, of an increase of output of Na*PG* of 2·5 mg./l. of urine (1·5 mg. pregnanediol/l.).

PLG in various urines. Some results are shown in Table 4.

PLG in the normal female. Fig. 2 shows the results of *PLG* estimation for more than a complete cycle in a woman (21 yr., recently married, nulliparous). Portions of 2 ml. of filtered extract (*F*) were used for entrainment.

Table 4. *PLG in human urine*

(*x*=ml. of filtered aqueous extract (*F*), of the final butanol residue used for entrainment.)

Urine	*PLG* (mg. Na*PG*/24 hr.) *x*=2	*x*=4
Pregnancy (9th month)	41·1	56·2
	38·1	66·6
Normal male	7·0	8·2
	6·0	13·2
	4·7	12·5
	5·0	14·3
Infant female (2·5 years)	1·8	2·6

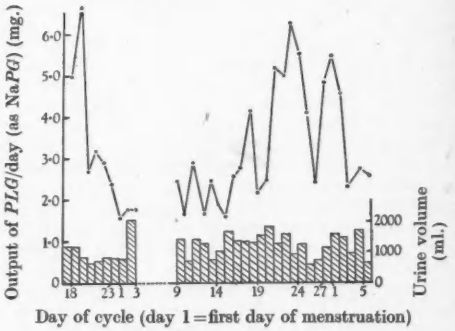

Fig. 2. *PLG* in female cycle.

All the above figures represent *minimal* values for *PLG*, because, as pointed out above, losses on butanol extraction and on entrainment cannot at present be estimated (except for Na*PG*) in the absence of precise knowledge of the properties of *PLG*.

DISCUSSION

The above results show that the method described will estimate Na*PG* with some accuracy at concentrations of about 2·5 mg./l. in urine in the presence of the rest of the *PLG* complex. The method, therefore, should be useful in the study of the human menstrual cycle, the 'luteal' phase of which we have had no difficulty in detecting, presumably because of the increased output of Na*PG* at this time. The method should also provide a rapid means of estimating *PLG* in late pregnancy, with small amounts of urine: further work would be needed to assess its value in early pregnancy. The conditions, other than pregnancy, in which *PLG* output may be increased include some forms of adrenal hypertrophy (cf. Venning, Weil & Browne, 1939; Salmon, Geist & Salmon, 1941) and, indeed, any condition in which

excretion of steroids conjugated with glucuronic acid is augmented.

With regard to the use of the term 'pregnanediol-like glucuronide (*PLG*)', it must be emphasized that this is a very vague conception at present, and can only be given precise significance when the results of investigations into the chemical nature of urinary glucuronide are available. At present, the most that can be said is that *PLG* presumably contains at least part of the constituents of Na*PG*(V), which may include the sodium glucuronidates of pregnane-3(α)-ol-20-one (Marrian & Gough, 1946) and of pregnane-3(α):17:20-triol (Mason & Kepler, 1945) as well as of pregnane-3(α):20(α)-diol itself. It is of course possible that *PLG* may be found to include glucuronides not of a steroid nature, although this would be surprising in view of its solubilities. It is certainly clear that *PLG* from urine from normal males may contain very little Na*PG*. According to Westphal (1944), the amount of pregnanediol is about 0·7 mg./l. (as barium pregnanediol glucuronidate), and we, ourselves, have found only traces in our *PLG* complex from this source.

One can vary the fraction of *PLG* isolated by altering the conditions of extraction and especially of entrainment: with sufficiently 'drastic' entrainment conditions one presumably includes much more soluble glucuronidates, and possibly even barium glucuronate itself, in the estimations. With our conditions, however, we are of the opinion that the *PLG* estimated closely resembles Na*PG* in its solubilities and preliminary isolation experiments have shown that it gives a considerable ether-soluble fraction after hydrolysis. We were especially interested in the work of Jayle & Libert (1946), who have isolated 'steroid glucuronides' by two entirely different methods and estimated them by the naphthoresorcinol reaction. Their results resemble our own quite closely and Jayle & Libert appear also to be dealing with glucuronide fractions similar to that which we call '*PLG*'.

Finally, it may be pointed out that our entrained material may prove to contain other conjugates (e.g. organic sulphates) from urine, and may be useful for isolation of such substances.

The chemical nature of the glucuronide fraction is being investigated, but in the meantime the method is reported in the hope that it may be of value in the examination of urinary output of glucuronidate.

SUMMARY

1. A method of estimating pregnanediol glucuronidate by entrainment on barium phosphate and subsequent application of a modified naphthoresorcinol reaction has been developed.

2. The method when applied to urine gives analytical figures for a glucuronide fraction which we have called 'pregnanediol-like glucuronide (*PLG*)'.

3. Minimal figures for *PLG* (as Na*PG*) in various urines, including those of the human menstrual cycle, are reported.

4. The nature of *PLG* is discussed.

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Relations between Potassium and Sodium Levels in Mammalian Muscle and Blood Plasma

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In a previous account of the question of ionic permeability of the muscle fibre membrane (Conway, 1946), two regulative mechanisms were considered: one, a differential and passive permeability whereby potassium and chloride entered freely, whereas ions such as sodium, magnesium, sulphate, etc. were excluded (or entered relatively very slowly), such permeability being determined by the ion size; the other, a possible mechanism whereby sodium ions if they entered very slowly and over a long period could be extruded by some active process.

For the first of these mechanisms the evidence already presented (Boyle & Conway, 1941; Conway, 1946) scarcely admits of any other interpretation in the case of isolated sartorius of the frog, and applies also to the proximal convoluted tubules of the frog's kidney (Conway, FitzGerald & MacDougald, 1946) and liver (Conway & Boyle, 1944). Wilde (1945) has shown mammalian muscle to be permeable to chloride, and many studies with isotopic potassium (e.g. Noonan, Fenn & Haege, 1941) have shown mammalian muscle to be freely permeable to potassium ions. Also, Shanes (1946) has presented evidence showing that spider crab nerve is freely permeable to potassium and chloride.

Concerning the second mechanism, there is no satisfactory evidence that sodium ions which have entered into skeletal muscle fibres are actively extruded. The small amount of the exchanges recorded with respect to the muscle tissue as a whole leaves it open to reasonable doubt as to the exchange of the sodium ions from *within* the fibres. An outstanding experiment of Heppel (1939), working in Fenn's laboratory, showed that when rats were fed on a diet with very low potassium content, sodium undoubtedly entered the muscle fibres, but experiments demonstrating the reversal of the process and its rate do not appear to have been carried out, though the restoration of the potassium levels was demonstrated (Heppel & Schmidt, 1938). The importance of this question for muscle physiology and the theory of ionic exchanges across the membranes of mammalian tissue cells led us to repeat these experiments, and to find whether extrusion of sodium occurred on returning the rats to a diet rich in potassium. Although our findings confirmed those of Heppel, the amount of sodium entering did not occur to the same extent, due possibly to the shorter time allowed on the low potassium diet; the duration of Heppel's experiments was about 45 days, ours lasting upwards of 30 days on the low potassium intake. We found an average increase from 53 to 80 mg. sodium/100 g. muscle after 30 days, 53 mg./100 g. being an average normal figure for the group of rats used in these experiments. After 24 days the sodium increased to *c.* 76 mg./100 g. The corresponding chloride changes left no doubt that much the greater fraction of this sodium had entered the fibres. The changes with respect to sodium on restoring such rats to a high potassium diet, as well as the concomitant potassium, chloride and water changes, are described below.

METHODS

Experimental. Young rats, averaging about 70 g. in weight, of the Wistar strain were used throughout. They were maintained on the low K diet for the required number of days, after which they were bled into small dry centrifuge tubes and the serum separated immediately and stored for analysis. For the muscle analyses, a small portion of the leg muscle (about 100 mg.) was removed into a weighed platinum crucible for subsequent ashing and Na and K determinations; larger amounts were required in the series in which muscle Cl analyses were carried out.

Diet. The low K diet consisted of the following (given as percentages by weight): glucose 52, casein 30, salad oil 10, cod-liver oil 1·6, wheat germ oil 0·7, 'ammonia' yeast (yeast in which all the K had been replaced by NH_4 ions (Conway & Breen, 1945)) 1·6, and salt mixture 4·1. The salt mixture was similar in composition to that used by Heppel (1939). The K content of this diet was 7–10 mg./100 g. and the average daily consumption of the diet/rat was about 10 g. The high K restoration diet contained (in g./100 g.): wheat flour 36, oat flour 36, casein 5, yeast 21, $CaCO_3$ 1, NaCl 1, with 0·17 KCl added (total K = 340 mg./100 g.). For a group of rats maintained for 28 hr. on the extra high K intake, a similar diet was used with KCl added to form 1 g. KCl/100 g. (total K 780 mg./100 g.).

Chemical. Na and K were determined by colorimetric modifications (Boyle, Conway, Kane & O'Reilly, 1941) of the methods of Salit (1932) and of Shohl & Bennett (1928) respectively, after ashing as described by Fenn & Cobb (1934). Serum Cl was determined by a microdiffusion method (Conway, 1939; 1947) and muscle Cl by a similar method after a preliminary diffusion into isotonic Na_2SO_4. The water content was found by drying overnight at 105° prior to ashing for Na and K determinations.

RESULTS

The mean results for the first series are shown in Table 1. It will be seen that an increase of Na in skeletal muscle occurs as in the experiments of Heppel. The increase is at first rapid and then more gradual, reaching approximately 36·5 mmol./kg. after 26 days. Prolongation of the dietary to 46 days, as for Heppel's rats, would probably give figures not far different from his. For the individual results there is a rather wide variability. Thus, after the 26th day, the three rats examined had 46·6, 34·4 and 28·7 mmol. Na/kg. Similar variability may be seen in the results of Heppel, who gives the pooled analyses from 2 or 3 rats; after 46 days the values ranged from 64·4 to 44·8 mmol. Na/kg. and the range for single rats would no doubt be larger.

Table 1. *Effect of low* K *diet on muscle* Na, K, Cl *and water content*

(Mean values are given.)

No. of rats	Days on low K diet	Muscle Na (mmol./kg.)	Muscle K (mmol./kg.)	Muscle Cl (mmol./kg.)	Muscle water (g./kg.)	Plasma Cl (mmol./kg.)
10	0	22·8	100·5	12·4	765	117·5
2	4	25·4	86·8	10·7	757	119
2	8	31·4	72·8	13·0	775	117
2	12	34·4	75·3	14·4	770	116
2	16	34·5	81·6	15·2	763	117
3	26	36·5	75·4	15·2	760	122

Table 2. *Changes of muscle and serum* Na *and* K *on transfer from low* K *diet to high* K *diet*

No. of rats	Days on low K diet	Muscle Na (mmol./kg.)	Muscle K (mmol./kg.)	Serum Na (mmol./kg.)	Serum K (mmol./kg.)	Muscle water (g./kg.)
10	0	22·8	100·5	141	4·9	765
15	27	33·2	82·4	142	3·6	760
	Days after change to high K diet					
6	1	33·5	102·6	143	5·5	—
5	2	33·1	101·0	146	6·9	—
5	3	26·5	98·2	148	7·6	—
5	4	26·5	100·7	146	8·0	—
5	6	23·9	105·1	144	8·7	—
5	16	21·7	102·0	144	9·0	—

The mean increase in the muscle Na was from 22·8 to 36·5 mmol./kg. whereas the Cl increased from 12·4 to 15·2 or an increase of 2·8 mmol./kg. Only a small fraction of the Na increase could therefore be interpreted as due to change in the interspaces between the muscle fibres; in short, the Na must have largely entered the fibres.

Another point of interest from Table 1 is that there is no immediate relation between the rise of Na in muscle and fall of K. After 26 days the K reduction is about 25 mmol. whereas the Na increase is only about 14. After 16 days the fall in K is 19 whereas the Na increase is only 12 mmol./kg.

In the second series of experiments conducted very shortly after the first and on similar rats, a group of 50 rats was placed on the low K diet as before. For this group, the 1 % NaCl was replaced by a salt mixture similar to Heppel's. The rats were maintained on this low K diet for 27 days, then 15 were taken and the leg muscles and serum analyzed. The remaining rats were placed on the K-rich diet (containing 340 mg. K/100 g.) of which they partook avidly. Table 2 shows that the muscle Na falls slowly to normal over a period of about 16 days, and then slightly below normal. After 24 hr., though the plasma K is well above the normal value and the muscle K restored, there is no significant change in the muscle Na. The same holds after the second 24 hr., the plasma K being now as high as 6·9 mmol./l. It is only after 3 days that a significant decrease is observed. After 6 days the muscle Na is near to the normal average value and after 16 days slightly below this level.

It was of interest to find if very high levels of K, rapidly produced, would increase the rate of Na return from the muscle. Some rats of the above group after the 27th day were fed on a diet with the K content raised by further KCl addition to 780 mg./kg. The rats ate considerable amounts of this food and after 28 hr. the plasma K was as high as 9·8 mmol./kg. The mean Na content of the muscles of these rats was 28·2 mmol./kg., which was still much higher than the mean 22·8 of the normal group, but lower than the corresponding 33·5 of the rats having 5·5 mmol. K/l. of plasma. It would appear, therefore, that for mammalian muscle very high plasma K had some influence in hastening the return of muscle Na from mammalian muscle.

With amphibian muscle at room temperature or in the cold, no evidence of the return of muscle Na, much increased by previous immersions in K-free Ringer, could be obtained when using high external K.

DISCUSSION

From the above results the following conclusions may be drawn.

(1) Mammalian muscle has the power of slowly extruding Na from within the muscle fibres.

(2) The extrusion of the Na is comparatively very slow compared with the rates of K entrance. Thus, from the work of Noonan *et al.* (1941), labelled K enters to half its equilibrium value in about 45 min. The return of the muscle Na when the plasma K is increased beyond the normal level becomes noticeable after about 3 days, and after about 1 day when the K is raised considerably.

(3) The process of the restoration cannot be interpreted as an antagonism between Na and K or a competition for acidic groups on the non-diffusible constituents, since almost immediately the muscle K was restored to its old level and the plasma K became even higher than normal, whereas for 48 hr. in the above experiments no sign of Na decrease occurred. The explanation must take account of the relative rates of passage of the ions to and from the fibres, and of a membrane alteration produced by very low K levels.

An interpretation of the ionic permeability of the muscle-fibre membrane in relation to the experimental findings

This is essentially that already put forward (Conway, 1946) in which two mechanisms controlling the normal K and Na entrance are postulated. The first, a permeability such that while K and Cl ions enter rapidly, Na enters comparatively very slowly, possibly many hundreds of times more slowly than K. The second, a slow, active extrusion of the very slowly entering Na ions. This interpretation is not inconsistent with the previous treatment of the physiological significance of inorganic levels in the internal medium (Conway, 1945).

It is further considered that when the plasma K falls below the normal level, the membrane is placed under increasing strain owing to the very high potential gradient across it. This strain may be likened to the dielectric strain of a condenser. As a result, Na ions enter the muscle more rapidly than usual. The question of such a change of permeability arising out of potential gradients across the membrane increased above a critical value has also been considered by Wilbrandt (1947) in a recent review. If we now consider an extrusion mechanism which, up to a point, returns Na ions to the plasma at a rate largely proportional to their concentration, then a steady state will develop with each level of plasma K. At normal K levels, the Na content of the muscle will be low, and when the plasma K falls to about half its normal level it will be relatively high. Similarly, with high plasma K the muscle Na, though low in concentration, will fall still lower, and this fall may be measurable. On such a view the Na extrusion rate need not alter, but only the entrance rate; at the same time the extrusion rate may alter considerably with the increased permeability of the membrane. The fact that in the restoration process the lowering of the high Na content of the muscle fibres is slow compared with the rate indicated by Heppel's experiments with radioactive Na, suggests either a marked reduction of the exit rate of Na on increasing the plasma K or a slow restoration of the membrane abnormality. It is also possible that the rate of mixing with the labelled ions does not measure the rate of ion transport (Ussing, 1947).

The entrance rate of sodium into the fibres in the low plasma potassium experiments of Heppel, compared with the normal entrance rate of potassium

Heppel found that radioactive Na interchanged freely with the Na which had entered the fibres. The figures suggested an even faster entrance rate than the normal K value, and was so referred to by Krogh (1946). This suggestion of a faster rate than K, however, is not borne out by closer examination, for it must be considered that Na is entering from a high external Na level into a relatively low concentration, whereas on the other hand K is entering from a very low external level into a high concentration. When these facts are taken into account, Na may be shown to enter the fibres under the abnormal conditions at only about one thirtieth the rate of the normal K entrance, as derived from the data of Noonan *et al.* (1941). How the K rate is affected by the abnormally low plasma K has not been shown, but we may suppose that it does not remain unaltered and is almost certainly increased, and possibly very much increased.

The relative rates as indicated above may be derived as follows: The rate (dQ_{mr}/dt) at which labelled K after injection accumulates in a muscle fibre may be expressed by the equation

$$\frac{dQ_{mr}}{dt} = (P_p C_{pr} - P_m C_{mr}) \times \text{surface}, \qquad (1)$$

where P_r and P_m are the entrance and exit rates respectively of K for the muscle fibre (per unit concentration and unit surface) under the given conditions, and C_{pr} and C_{mr} are labelled K concentrations in plasma and muscle respectively. For the relatively short half period for equilibration the potential across the membrane may be taken as

constant. Also, it may be noted that it has been shown (Boyle & Conway, 1941; Hill & Kupalow, 1930) that all, or practically all, the K in the muscle fibre is ionized and not bound to protein, so that the difference between P_r and P_m may be held to reflect only the effect of the potential difference within and without the fibre. To bring these P_r and P_m values more into relation with permeability figures as ordinarily expressed, and to Fick diffusion constants, it may be assumed that the diffusion from the outer to the inner medium occurs into zero concentration, and similarly for the inner to outer diffusion. In the steady state dQ_{mr}/dt is zero.

If the labelled K in the plasma is constant or nearly so during the half period of equilibration, it may be deduced from equation 1 that

$$P_p = \frac{0{\cdot}276r}{t_{0{\cdot}5}} \times \frac{C_m}{C_p}, \qquad (2)$$

where r is the radius of the fibre (0·8 of which is present as 'fibre water') and $t_{0{\cdot}5}$ is the half period of equilibration. Writing a similar equation for labelled Na under the same conditions and dividing we obtain

$$P_p/P'_p = \frac{t'_{0{\cdot}5}}{t_{0{\cdot}5}} \times \frac{C'_p}{C'_m} \times \frac{C_m}{C_p} \qquad (3)$$

in which the dashed symbols represent the values for Na. If we compare the entrance rate of labelled Na in Heppel's experiments with the entrance rate of labelled K into normal rats, then the concentration ratio of K in the fibre to K in the plasma is practically the same (in Heppel's data) as the normal ratio, and the potential difference also may thus be taken as the same. In such experiments of Heppel, the Na very probably enters at a more rapid rate than for normal fibres, seeing that Na has entered the fibres to a considerable extent, but if we suppose it to enter at the same rate, then the conditions of comparison would be the same or very similar.

Allowing for the interspace Na, it appears from Heppel's data that the half period of exchange of the labelled Na is approximately 15 min. Throughout this period the serum counts for the labelled Na do not change appreciably with time. From Noonan, Fenn & Haege's data the half period of the labelled K entrance in normal rats is of the order of 30–60 min. 45 min. may be taken as probably not far from the real figure, and it would appear from the data as a whole that there was no considerable fall in the labelled K in the plasma, the radioactive material being injected mainly intraperitoneally and for some observations, subcutaneously. It may be assumed then that the ratio of the half periods for the labelled Na equilibration and that of labelled K in such experiments is about three to one, so that

$$P_p/P'_p = 82/3 = 27,$$

i.e. the entrance rate of Na ions into the muscle under strain with the abnormally low plasma K is only about one thirtieth the rate of the entrance of K ions into the muscle under normal conditions. It may therefore be concluded that the entrance rate of Na ions into mammalian muscle in the living animal, and under conditions of membrane strain, is considerably less than the entrance rate of K ions, and for muscle under normal conditions it may be only a very minute fraction.

It would seem rational to suppose that a regulating system for the muscle fibre which would allow K ions to pass freely, but exclude Na ions without energy expenditure, would be more advantageous than one requiring energy for the extrusion of the Na ions which freely entered. The formation of a membrane by the cell or muscle fibre which would achieve this *perfectly*, and at 37°, is perhaps impossible, but it would appear that a close approximation to it can be achieved, whereby the energy requirement for Na extrusion approaches near to zero. It may also be added that in numerous experiments we have been unable to detect any extrusion rate of Na ions from the frog's sartorius in the cold.

SUMMARY

1. Sodium which had entered the muscle fibres of rats, after a period of potassium deprivation, was slowly extruded when the rats were placed on a diet rich in potassium. The mean half period of extrusion, even with the plasma potassium above the normal level after 24 hr., was about 3 days.

2. The muscle potassium, which had fallen to levels of 75–82 mmol./kg. in the average, was fully restored to normal values (about 100 mmol./kg.) after 1 day on the high potassium diet, and did not appreciably change from this when the plasma concentration reached 9·0 mmol./kg.

3. Considerable variability was shown by individual rats in the amounts of sodium which had entered the muscle fibres during potassium deprivation.

4. Examination of the data of Heppel (1940) for the rate of mixing of labelled ions with the sodium which had entered the fibres during the low potassium period shows that the true entrance rate of potassium/unit concentration into such fibres is about 30 times as great as that of sodium.

5. The relative rate of passage of potassium ions into the normal fibres may be far greater than this, as evidenced by the comparatively very slow extrusion of muscle sodium, and it is also possible, as suggested by Ussing (1947), that the rate of mixing of the labelled ions may give values which are too high for ion transport.

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The Microbiological Degradation of Steroids

4. FISSION OF THE STEROID MOLECULE

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During recent years the presence of an enzyme system capable of effecting the oxidation of hydroxysteroids to the corresponding keto derivatives has been clearly demonstrated in a number of bacterial species. The investigations of the Italian schools of Ercoli and Arnaudi have resulted in the isolation from yeast of several new organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1939), *F. androstenedionicum* (Ercoli & Molina, 1944), and *F. carbonilicum* (Molina & Ercoli, 1944), which, in general, oxidize the nuclear hydroxyl groups of steroid compounds devoid of the 17 side chain. Zimmermann & May (1944) obtained similar oxidations by the action of pseudodiphtheria bacilli. In the bile acid series stepwise oxidation of the hydroxyl groups has been achieved using *Alcaligenes faecalis* (Schmidt, Hughes, Green & Cooper, 1942), and more recently, *Escherichia coli* (Schmidt & Hughes, 1944). The independent investigations of the flora of soils by Tak (1942), and by the present author (Turfitt, 1943, 1944*a*), have shown that the cholesterol-decomposing organisms fall essentially within the genus *Proactinomyces*. These organisms are of particular interest in the breakdown of steroids since their oxidizing action is not, as reported for other organisms, inhibited completely by the presence of the intact side chain. Their place in the economy of nature is discussed later.

Whilst this primary oxidation of steroid hydroxyl groups is firmly substantiated, actual breakdown of the steroid molecules, yielding distinct chemical fission products, has not hitherto been recorded. Evidence of a secondary action of this nature has, however, been obtained during large-scale work on the oxidation of cholesterol to cholest-4-en-3-one (Turfitt, 1944*b*). With cholesterol as the sole carbon source, energy for growth must inevitably, in view of the non-autotrophic character of *Proactinomyces*, have resulted from utilization of the sterol carbon. During the course of the work certain small fractions were obtained which undoubtedly represented products of degradation, but attempts to isolate definite compounds were unsuccessful. A detailed account of the various methods by which information has been gained as to the course of the degradation process is given in the present publication.

EXPERIMENTAL

Foci of bacterial attack

Fission of the steroid skeleton by bacterial oxidation might be expected, by analogy with chemical methods, to occur primarily at one of several susceptible points, viz. OH groups, double bonds, or the side chain. With a view to determining whether the presence or absence of each of these points exerts any material effect upon the bacterial oxidation, a series of compounds covering different combinations of OH, double bond and side chain has been subjected to the oxidizing action of *P. erythropolis*.

In each experiment, performed in triplicate, 50 mg. finely powdered, sterile compound were introduced with aseptic

precautions into a 250 ml. conical culture flask containing 100 ml. mineral salt solution, previously sterilized at 115° for 15 min. (The mineral salt solution referred to throughout this publication had the following composition: NH_4NO_3, 0·1; K_2HPO_4, 0·025; $MgSO_4.7H_2O$, 0·025; NaCl, 0·0005; $FeSO_4.7H_2O$, 0·00001%.) Inoculation was performed from 24 hr. agar-slope cultures of *P. erythropolis* (strain 15/G), and the cultures incubated at 25°. At intervals of 4, 7 and 11 days test samples of the cultures were treated with Universal Indicator (British Drug Houses Ltd.), production of acid being taken as indicating steroid decomposition.

A summary of the results of these experiments is given in Table 1. With those compounds in which the side chain is shortened either partially or completely to >CO, degradation is apparently more rapidly initiated. When, on the other hand, the hydrocarbon side chain is intact, blocking of the hydroxyl group or of the double bond, or of both, does not prevent attack, although it seems to occasion some slight delay. Unless, therefore, there exists some other unsuspected point of oxidative attack, this would appear to be presumptive evidence of side-chain disruption.

Table 1. *Steroid utilization by* P. erythropolis

Compound	Points available			Universal indicator		
	—OH	Double bond	Side chain	4 days	7 days	11 days
Cholesterol	+	+	+	YR*	R	R
Stigmasterol	+	+	+	YR	R	R
Sitosterol	+	+	+	YR	R	R
Coprosterol	+	−	+	Y	R	R
Dihydrocholesterol	+	−	+	Y	R	R
Cholesterol acetate	−	+	+	YR	R	R
Coprosterol acetate	−	−	+	Y	R	R
Cholestane	−	−	+	Y	R	R
3-Hydroxychol-5-enic acid	+	+	+ (modified)	R	R	R
*trans*Dehydroandrosterone	+	+	−	R	R	R
Androsterone	+	−	−	R	R	R
Androsterone acetate	−	−	−	R	R	R

* R = Red (pH < 4); Y = Yellow (pH 6·5); reaction of original medium = Green (pH ~ 8).

Attempted isolation of volatile products

During the classical work on sterol structure two volatile materials were isolated which had resulted from oxidative disruption of the side chain, viz. acetone, originating from the terminal *iso*propyl group (Windaus & Neukirchen, 1919), and methyl *iso*hexyl ketone representing removal of the complete side chain (Windaus & Resau, 1913). Attention has thus first been directed towards determining whether the *Proactinomyces* oxidation proceeds along a similar course with the production of volatile ketones or possibly laevulaldehyde.

A 1 l. conical flask containing 250 ml. mineral salt solution and 2·5 g. $CaCO_3$ was autoclaved at 115° for 15 min., and after cooling 2 g. finely powdered sterile coprosterol acetate were added. The flask and contents were inoculated with *P. erythropolis* (strain 15/G) and incubated at 25° for 2 months, during which time sterile O_2 was slowly bubbled through the culture and thence through 25 ml. saturated solution of *o*-nitrobenzaldehyde in 2N-NaOH contained in a 100 ml. tube. At the end of 2 months the contents of the tube were warmed for 5 min. on the water bath, cooled and extracted with 5 ml. chloroform; the red or blue colour characteristic of the $CH_3.CO$— grouping under these conditions was not obtained. The experiment was repeated with like result using cholestane as substrate. Superficially this would appear to indicate that during this steroid decomposition volatile ketones and aldehydes are not produced, and in particular that the side chain is neither partially nor completely removed in the manner encountered in chemical oxidations. The results, however, are equally consistent with the theory that volatile compounds are produced, and are at once utilized by the organisms. In support of this theory it has been found by cultural experiments that *P. erythropolis* readily utilizes acetone, methyl *iso*hexyl ketone and laevulaldehyde when each of these substances constitutes the sole available carbon source in the culture medium.

Degradation of cholest-4-en-3-one

A quantitative study has been made of the production of cholest-4-en-3-one from cholesterol by *Proactinomyces* oxidation, and of its subsequent decomposition.

Six 250 ml. conical culture flasks, each containing 100 ml. mineral salt solution and 0·5 g. $CaCO_3$, were autoclaved at 115° for 15 min., and into each flask were introduced aseptically 50 mg. finely powdered cholesterol previously sterilized by steaming in a sealed tube. After inoculation from 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G), the flasks were incubated at 25° for periods of $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3 and 4 months respectively. After the appropriate incubation period, each culture was removed and thoroughly extracted with chloroform; the extract was washed, dried with anhydrous Na_2SO_4 and evaporated. Curve *A* (Fig. 1) shows the decrease in total chloroform-soluble material throughout the culture period. The residues were redissolved in chloroform. Residual cholesterol (curve *B*) was determined by the method previously described (Turfitt, 1943), and 3-ketosteroids (curve *C*) by the Callow modification (Callow, Callow & Emmens, 1938) of the original Zimmermann procedure (Zimmermann, 1936). In order to

determine decomposition products present as either soluble or insoluble Ca salts, the aqueous suspension from the initial chloroform extraction was acidified with diluted HCl, and re-extracted with chloroform; at no time, however, was more than 1·5 mg. residue obtained from the extracts. The total chloroform-soluble material was accounted for almost entirely by cholesterol + cholest-4-en-3-one, and it was evident that degradation products of cholest-4-en-3-one only existed in the cultures in minute amounts.

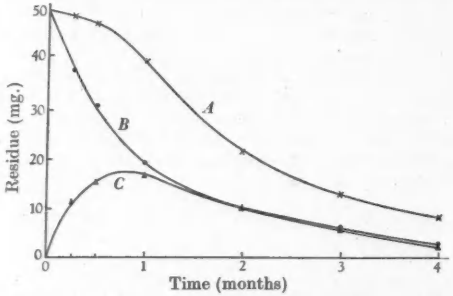


Fig. 1. Variation of total chloroform-soluble material (curve *A*), cholesterol (curve *B*), and 3-ketosteroids (curve *C*) in culture of *P. erythropolis* on cholesterol.

In an endeavour to isolate individual breakdown products in sufficient quantity for characterization, a considerable number of large-scale experiments was tried, each using 200 g. cholest-4-en-3-one prepared chemically from cholesterol by the Oppenauer oxidation (Oppenauer, 1941). In spite of the wide variety of experimental conditions no success was achieved, and it became increasingly apparent that the primary products of fission of the molecule were themselves attacked by the organisms in preference to the parent steroid, and that trapping of any acidic products as Ca salts did not prevent their further breakdown.

Continual removal of the breakdown products from the sphere of action of the organisms appeared to be the only logical method of isolating any intermediates. No suitable process for the purpose could be found recorded in the literature, and a technique was finally perfected employing the continuous culture apparatus shown in Fig. 2, which was kindly constructed to the author's specification by Messrs Quickfit and Quartz, King's Norton, Birmingham. Finely powdered sterile cholest-4-en-3-one (20 g.) was introduced into the previously autoclaved 2 l. reaction vessel *A*, which was then connected to the sterile unit *B* containing 3 l. mineral salt solution, and to the receiver units *U* and *C*. With the tap *T* open, culture medium was allowed to siphon into *A*, and a heavy inoculum of *P. erythropolis* (strain 42/G) was introduced. A fresh supply of sterile medium was connected to vessel *A*, and the tap adjusted so that the rate of flow from the nozzle *N* was approximately 1 drop/sec. Three litres of fresh culture medium were thus admitted during approx. 24 hr. Throughout the period of incubation (2 months) the reaction vessel was immersed in a water bath maintained at 25°. Outflow of medium occurred via the side arm *S* where loss of solid particles of steroid was prevented by the loose-fitting glass-wool plug. Sterile O_2 was passed continuously through the medium at the rate of approx. five bubbles/min., whilst the particles of the sediment at the bottom of the reaction vessel were gently agitated at intervals of a few hours. Further bacterial activity in the effluent was prevented by allowing the issuing drops to pass through a 1 in. layer of ether in the unit *U*, the ether being replenished from time to time to maintain this layer. The ether-saturated effluent was collected in the 3 l. flask *C*.

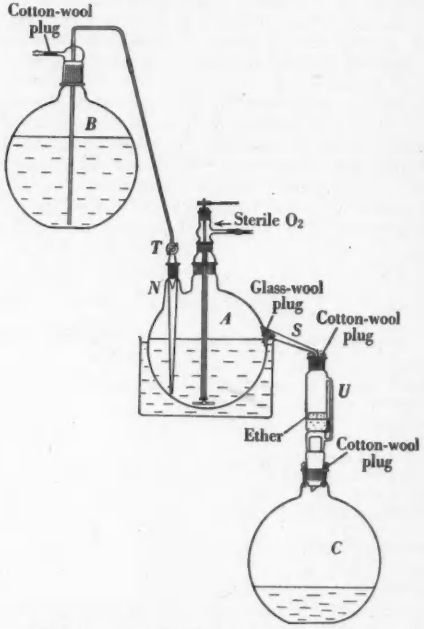


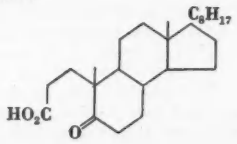
Fig. 2. Apparatus for continuous culture and collection of products.

After 2 months' incubation the assembly was dismantled, the contents of the reaction vessel filtered, and the filtrate added to the pooled effluents; the residue (A) was dried in an air oven at 25°. By evaporation under reduced pressure of the ether-saturated spent medium, there were obtained 500 ml. of a pale straw-coloured liquid with a heavy granular deposit (B).

The detailed examination of the fractions A and B is given schematically in Figs. 3 and 4.

Characterization of products III and IV

Product III. On standing, the gum deposited rosettes of white needles. By repeated fractional crystallization from light petroleum (b.p. 100–120°) two products were obtained: (*a*) 4 mg. white plates, m.p. 150°. A specimen of the keto-acid of the formula



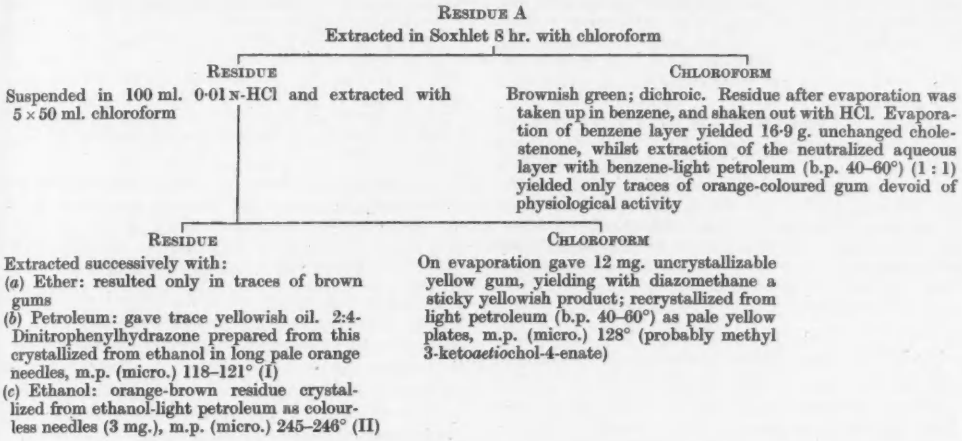

RESIDUE A
Extracted in Soxhlet 8 hr. with chloroform
RESIDUE
Suspended in 100 ml. 0·01 N-HCl and extracted with 5 × 50 ml. chloroform
CHLOROFORM
Brownish green; dichroic. Residue after evaporation was taken up in benzene, and shaken out with HCl. Evaporation of benzene layer yielded 16·9 g. unchanged cholestenone, whilst extraction of the neutralized aqueous layer with benzene-light petroleum (b.p. 40–60°) (1 : 1) yielded only traces of orange-coloured gum devoid of physiological activity
RESIDUE
Extracted successively with:
(a) Ether: resulted only in traces of brown gums
(b) Petroleum: gave trace yellowish oil. 2:4-Dinitrophenylhydrazone prepared from this crystallized from ethanol in long pale orange needles, m.p. (micro.) 118–121° (I)
(c) Ethanol: orange-brown residue crystallized from ethanol-light petroleum as colourless needles (3 mg.), m.p. (micro.) 245–246° (II)
CHLOROFORM
On evaporation gave 12 mg. uncrystallizable yellow gum, yielding with diazomethane a sticky yellowish product; recrystallized from light petroleum (b.p. 40–60°) as pale yellow plates, m.p. (micro.) 128° (probably methyl 3-keto*aetio*chol-4-enate)

Fig. 3. Fractionation of solid residue from degradation of cholest-4-en-3-one.

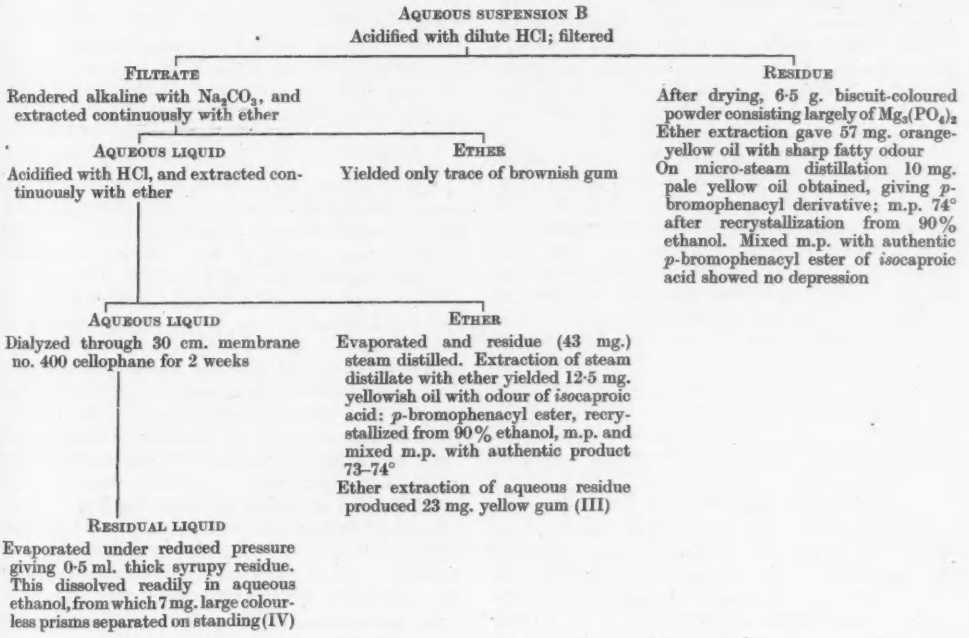

AQUEOUS SUSPENSION B
Acidified with dilute HCl; filtered
FILTRATE
Rendered alkaline with Na_2CO_3, and extracted continuously with ether
RESIDUE
After drying, 6·5 g. biscuit-coloured powder consisting largely of $Mg_3(PO_4)_2$
Ether extraction gave 57 mg. orange-yellow oil with sharp fatty odour
On micro-steam distillation 10 mg. pale yellow oil obtained, giving *p*-bromophenacyl derivative; m.p. 74° after recrystallization from 90% ethanol. Mixed m.p. with authentic *p*-bromophenacyl ester of *iso*caproic acid showed no depression
AQUEOUS LIQUID
Acidified with HCl, and extracted continuously with ether
ETHER
Yielded only trace of brownish gum
AQUEOUS LIQUID
Dialyzed through 30 cm. membrane no. 400 cellophane for 2 weeks
ETHER
Evaporated and residue (43 mg.) steam distilled. Extraction of steam distillate with ether yielded 12·5 mg. yellowish oil with odour of *iso*caproic acid: *p*-bromophenacyl ester, recrystallized from 90% ethanol, m.p. and mixed m.p. with authentic product 73–74°
Ether extraction of aqueous residue produced 23 mg. yellow gum (III)
RESIDUAL LIQUID
Evaporated under reduced pressure giving 0·5 ml. thick syrupy residue. This dissolved readily in aqueous ethanol, from which 7 mg. large colourless prisms separated on standing (IV)

Fig. 4. Fractionation of fluid product of degradation of cholest-4-en-3-one.

prepared by the original method of Windaus (1906) gave no depression of the melting point in admixture with this product. (*b*) 9 mg. slightly yellow crystals, m.p. 245°. Methyl derivative, prepared by means of diazomethane, and crystallized from light petroleum (b.p. 40–60°), had m.p. 130°, not depressed when mixed with methyl 3-keto-*aetio*chol-4-enate.

Product IV. The prisms, m.p. 96–98°, were readily soluble in water, the solution being neutral to litmus, slightly soluble in 90% ethanol, and insoluble in ether, chloroform and light petroleum. N, S, P and halogens were absent. On heating, the product melted and then charred with a 'burnt-sugar' odour. The solution did not reduce Fehling's solution, but gave a positive Molisch test. A red colour was obtained by heating with resorcinol in HCl, but no reaction was obtained with phloroglucinol in HCl. 2:4-Dinitrophenylhydrazine also gave a negative response.

Owing to the small yield of product no further attempts to characterize the material were possible. At present, speculation as to the origin of this product is somewhat premature, but if it should be found to be a genuine product of cholestenone degradation it would produce a most valuable clue to the ultimate fate of the ring structure. The likelihood is, however, that it is a product of synthesis, a view which would seem to be supported by the fact that carbohydrates are not in general susceptible to utilization by *Proactinomyces* spp.

The products isolated and characterized as a result of the above process demonstrate the course of the initial fission of the cholestenone molecule under *Proactinomyces* attack. *iso*Caproic acid and 3-keto-*aetio*chol-4-enic acid are obviously complementary products of the fission of the side chain at C_{20}–C_{22}, the 21–CH_3 being lost completely. This evidence of the removal of the entire side chain affords an explanation of the failure to isolate the intermediates, 3-ketochol-4-enic acid, 3-keto*nor*chol-4-enic acid and 3-keto*bisnor*chol-4-enic acid which would be expected to result from a stepwise breakdown of the side chain. A simultaneous rupture of ring *A* is indicated by the production of the keto acid already known to result from hypobromite oxidation of cholestenone. The extreme smallness of the yields fully justifies the theory that the initial breakdown products are themselves rapidly utilized, so that the unidentified materials (I) and (II) must be taken either to represent secondary degradation products, or to contain products derived from the simultaneous oxidations at the two susceptible points.

Degradation of 3-ketochol-4-enic acid

The use of 3-hydroxychol-4-enic acid as substrate in *Proactinomyces* cultures leads to a large and rapid increase in bacterial population, and the fall in pH of the medium occurs somewhat earlier than in the case of cholesterol. Since the structure of the primary oxidation product, 3-ketochol-4-enic acid, is essentially that of cholest-4-en-3-one, apart from the modified side chain, further oxidation should follow a similar course in both compounds.

A quantity of 10 g. of 3-ketochol-4-enic acid was treated in the continuous culture apparatus as described under cholestenone, and the 'spent' medium and residual solid substrate carried through an identical extraction process. A weight of 8·7 g. of unchanged 3-ketochol-4-enic acid was recovered, and 3-keto*aetio*chol-4-enic acid isolated in 7 mg. yield.

No conclusive evidence of a fission of ring *A* could be obtained, since the only other materials isolated consisted of uncrystallizable yellowish brown gums.

Degradation of cholesterol acetate

Previous experience with cholesterol acetate as substrate has been that, whilst the bacterial count increases and acid is produced, the steroid is largely recovered unchanged. From this it has been assumed that *Proactinomyces* possess no enzymic mechanism capable of hydrolyzing the esterified linkage, thus permitting oxidation of the liberated OH group.

A quantity of 20 g. cholesterol acetate has been treated according to the process described above, incubation in this instance being continued for a total period of 3 months. Even after this time 18·9 g. of the initial material were recovered unchanged. *iso*Caproic acid was obtained in 10 mg. yield, indicating a disruptive oxidation of the side chain, but repeated attempts to isolate a crystalline product from the 26 mg. of brownish gum expected to contain the acetate of 3-hydroxy*aetio*chol-5-enic acid were unsuccessful.

Since no evidence of any kind was obtained in this experiment of an actual ring fission, it still appears that in the case of cholesterol acetate there is but a single focus of initial bacterial attack.

Degradation of 3:7:12-triketocholanic acid

In the oxidation of cholic acid by *Alcaligenes faecalis* (Hoehn, Schmidt & Hughes, 1944) stepwise oxidation of the three hydroxyl groups occurred, the 3(α)-OH being oxidized last, and 3:7:12-triketocholanic acid was obtained as end product in 85% yield. The general method and detailed isolation technique introduced by these workers has been followed in an investigation of *Proactinomyces* attack on cholic acid, and essentially similar results have been obtained. The further, disruptive oxidation of 3:7:12-triketocholanic acid is of considerable interest, since each of the three keto groups should theoretically provide a susceptible point for bacterial attack, in addition to the probable attack on the shortened side chain. A complicating factor, however, from the cultural standpoint is the greater solubility of cholic acid and the fact that growth of *Proactinomyces* is inhibited in concentrations above approximately 1500 mg./100 ml.

The continuous culture process described in detail above was employed in slightly modified form. Initially, the empty reaction vessel *A* was autoclaved and connected with the

units *B* and *UC*, 10 ml. water containing the combined surface growth of three 24 hr. agar-slope cultures of *P. erythropolis* (strain 42/G) being subsequently introduced into the reaction vessel. The flask *B* and contents, 3 l. mineral salt solution + 1·0 g. cholic acid, sterilized by autoclaving 5 min. at 115°, were replaced as required in order to maintain a continuous flow of solution at approx. 1 drop/sec. After 2 months' incubation, culture medium containing 52 g. cholic acid had been utilized. The spent medium, including the final contents of the reaction vessel, was evaporated under reduced pressure to 1 l. The resulting orange-coloured concentrate was rendered alkaline with ammonia and filtered. Solvent extraction of the residue (A) did not yield any identifiable product, neither was any material other than traces of brown gum obtained by continuous extractions of the residue, after suspension in dilute HCl, with light petroleum (b.p. 40–60°) and ether. The filtrate (B) was acidified with dilute HCl, and cooled with ice. On standing, the heavy, granular precipitate became crystalline, and was filtered off and dried (27·3 g.). After recrystallization from ethanol this product had m.p. 234°; in admixture with authentic 3:7:12-triketocholanic acid (m.p. 236°), a m.p. of 228° was obtained. The crystalline product was accordingly examined for the presence of intermediate oxidation products by the method described by Hoehn *et al.* (1944), but neither cholic acid nor any hydroxyketocholanic acid was isolated. The product was dissolved in 60 ml. methanol, 1 ml. conc. H_2SO_4 added, and the solution gently refluxed for 15 min. No precipitate was obtained by cooling in ice, but on addition of 10 ml. water followed by further ice cooling for 24 hr., a deposit of rosettes of colourless needles was obtained, m.p. 222°. Repeated recrystallization from aqueous ethanol only raised the m.p. to 228°, which was depressed to 218° in admixture with a specimen of methyl 3:7:12-triketocholanate. The product was hydrolyzed and the free acidic materials regenerated. Repeated fractional crystallization from ethanol-acetone-ether yielded 15·7 g. 3:7:12-triketocholanic acid, m.p. 234°, and 19 mg. of a crystalline acid, m.p. 240°. Further recrystallizations of this latter product from acetone-ether did not raise the melting point. In admixture with authentic 3:7:12-triketo-*allo*cholanic acid, m.p. 245°, no depression was observed.

The isolation of this product of degradation points to the side chain as the primary focus of attack on the molecule, and a careful search has failed to isolate any product arising from rupture of the ring system.

Oxidation of α-oestradiol

Proactinomyces oxidations in the steroid group have hitherto been concerned solely with compounds of saturated ring structure. In view of the readiness with which these organisms utilize simple aromatic compounds such as phenol and naphthalene (Gray & Thornton, 1928), it appeared probable that the benzenoid ring structure in steroid compounds would present no obstacle to *Proactinomyces* attack. In several small-scale experiments with *P. erythropolis*, however, whilst there was some slight increase in bacterial numbers, the pH of the medium remained unchanged, and the whole of the starting material was recovered unaltered. These unexpected initial failures were at first attributed to a diminution in the oxidative capacity of the particular strain used, but similar lack of success was encountered with typical strains of all the known *Proactinomyces* species. A more detailed study of the process was accordingly made as follows.

Finely ground sterile α-oestradiol (100 mg.) was introduced aseptically into 250 ml. sterile mineral salt solution contained in a 500 ml. conical culture flask. An extremely heavy inoculum, the growth from twelve 24 hr. agar-slope cultures of *P. erythropolis* (strain 143/N), was added. The culture was incubated at 25° for 2 months, during which time the flask was gently rocked, and a steady stream of sterile oxygen was bubbled through the culture. After the incubation period the medium was filtered, and the residue (A) dried in an air oven at 25°. The filtrate (B) was extracted successively with light petroleum (b.p. 40–60°), ether and chloroform, and this extraction was repeated after acidification of the aqueous liquid with dilute H_2SO_4; evaporation of the extracts yielded only minute amounts of brown resinous gums. The dried residue (A) was extracted continuously with chloroform in a Soxhlet for 3 hr., and the extract evaporated to dryness under reduced pressure. The residue was dissolved in 15 ml. 75% ethanol, and shaken with three successive 5 ml. quantities of light petroleum (b.p. 40–60°). The ethanol phase was separated, and examined for unchanged α-oestradiol and for oestrone by a modification of the technique described by Szego & Samuels (1943). On evaporation under reduced pressure 97·3 mg. almost colourless residue was obtained. This was dissolved in 20 ml. 15% acetone-light petroleum (b.p. 40–60°) and run on to a column (10 × 1 cm.) of 'adsorption alumina' (British Drug Houses Ltd.) previously treated with the same solvent. The column was washed with 80 ml. of acetone-light petroleum (b.p. 40–60°) mixture. The eluate was evaporated to dryness on a water bath, and the crystalline residue dissolved in 10 ml. ethanol, a separation of ketonic and non-ketonic materials being effected by the Girard (T) technique. The non-ketonic fraction consisted of 85·6 mg. almost pure α-oestradiol. Since the 5·5 mg. of brownish ketonic fraction could not be induced to crystallize an attempt was made to prepare a semicarbazone. 3·1 mg. long, colourless needles were thus obtained, and recrystallized from aqueous ethanol. No depression of the m.p. (256°, micro.) was observed in admixture with a prepared specimen of the semicarbazone of oestrone.

This experiment has been repeated with other *Proactinomyces* spp., but in no case has any actual degradation product been isolated, and oxidation at C_{17} with formation of oestrone is detectable only when the bacterial population is abnormally high. Ercoli (1941) has already reported a similar experience with *Micrococcus dehydrogenans*.

During this work on α-oestradiol the interesting observation was made that many *Proactinomyces* strains, after prolonged culture upon the diol as sole carbon source, show a diminution in their capacity for oxidizing cholesterol and other steroids with the intact 17 side chain; typical examples are given in Table 2.

There is no immediately apparent explanation of this phenomenon, and the fact that it is not

Table 2. *Diminution of steroid-decomposing capacity*

Strain	Cholestenone from 100 mg. cholesterol; 2 months incubation: Before oestradiol culture (mg.)	After oestradiol culture (mg.)
P. erythropolis M 14	20	17
P. erythropolis M 18/G	25	9
P. erythropolis 42/G	31	23
P. globerulum H/53	17	12
P. aquosus T 5/G	29	20
P. aquosus T 29/G	12	8

encountered, or only in small degree, with various other *Proactinomyces* strains renders it almost impossible, without further cultural study, to formulate even a tentative hypothesis in explanation.

DISCUSSION

With the accumulation of data on the process of steroid degradation by the microflora of soils, the fundamental importance of these organisms in the economy of nature becomes apparent. The mechanism of the synthesis of the polyhydro*cyclo*pentenophenanthrene compounds, both in the animal and vegetable kingdoms, is still obscure, and the complex processes of metabolism, resulting in the biogenesis of the sex hormones and other similar products, has still to be finally elucidated. These processes of synthesis, however, and the subsequent bacterial disposal of the steroid materials when divorced from the living organism may be regarded as constituting a natural 'steroid cycle'. Decomposition of steroids deposited in soils is, in general, a relatively rapid process, although in adverse environmental conditions bacterial activity is restricted and some steroid accumulation results.

In the oxidation of sterols with the 17 side chain intact *Proactinomyces* still appears to occupy a unique position, but a detailed investigation (Turfitt, 1947) of the organisms utilizing other steroids where the side chain is not present indicates that various Gram− organisms may also play a not inconsiderable role in the disposal of this type of compound, whilst moulds and actinomycetes are more or less inactive in this respect.

The products of steroid breakdown do not, so far as they have been individually isolated and investigated, appear to have any significance as plant growth stimulants. The possibility of phytohormone activity in the further disruptive products has been examined experimentally in the case of the germination of cress seeds, and also in the root formation and growth promotion of lettuce and tomato seedlings. Two methods were investigated in this connexion; in the first method, cholesterol, carefully purified via the dibromide, was intimately mixed with the experimental soil, and the seeds or seedlings watered daily with a suspension of *P. erythropolis*. In the second technique, no steroid was initially added to the soil, but the latter was treated with a heavy aqueous suspension of *P. erythropolis*; subsequently, watering was carried out with aqueous solutions or suspensions of the crude isolates from the continuous culture method. Comparison with the corresponding controls, however, revealed no indication of stimulant action. In the absence of any contrary evidence, therefore, it must provisionally be considered that bacterial decomposition of steroids is essentially a process for the disposal of waste products.

SUMMARY

1. Further *Proactinomyces* oxidation of steroid ketones, obtained as primary oxidation products of hydroxy steroids, results in disruption of the molecules.

2. Yields of degradation products are small, due largely to their further bacterial decomposition. By a continuous culture process sufficient individual products may generally be isolated to permit of identification, but the bacterial oxidation is in no way comparable with chemical technique as a preparative method.

3. Two types of oxidation are encountered, and are frequently simultaneous processes, viz. ring fission at >CO group, and complete removal of side chain giving the *aetio*carboxylic acid.

4. Thus, cholest-4-en-3-one yields 3-keto*aetio*chol-4-enic acid and *iso*caproic acid and Windaus's keto acid by disruption of ring *A* at C_3–C_4.

5. Blocking of free hydroxyl at C_3, as in cholesterol acetate, apparently prevents bacterial fission of the ring at this point, but *iso*caproic acid is again obtained from degradation of the side chain. The expected complementary product, the acetate of 3-hydroxy*aetio*chol-5-enic acid, has not been isolated.

6. Side-chain fission of 3-ketochol-4-enic acid yields 3-keto*aetio*chol-4-enic acid, but no definite product has been isolated as evidence of fission at the 3-keto group.

7. The side chain is also completely removed from 3:7:12-triketocholanic acid giving 3:7:12-triketo-*aetio*cholanic acid, but again there is no conclusive evidence of ring fission at any of the susceptible points.

8. In α-oestradiol the benzenoid ring appears to have an inhibiting influence on the bacterial dehydrogenase system, and it is only when the bacterial population is abnormally high that some slight oxidation to oestrone occurs. No evidence of actual molecular degradation has been obtained.

9. Review of available information suggests the presence of a natural 'steroid cycle', degradation of steroids by soil microflora apparently providing a disposal mechanism for waste materials.

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The Mechanism of the Effect of X-rays on Bacterial Toxins

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PART 1. THE SENSITIVITY OF TOXINS TO X-RAYS

Relatively little work has so far been done on the effect of X-rays on bacterial toxins. Gerhartz (1909) studied the effect of X-rays on diphtheria toxin, and Fabre & Ostrovsky (1912) found a partial inactivation of this toxin following the addition of radium to the toxin solution. Ferroux & Muttermulch (1925) detoxified tetanus toxin with radium and observed that the loss of toxicity was accompanied by a loss of antigenicity. Baker (1935) showed that the sensitivity of tetanus toxin to radium was similar to that of Rous sarcoma virus and bacteriophage, and was less marked than the sensitivity of bacteria. Carpenter & Stokinger (1942), working with culture filtrates of *Clostridium botulinum*, *Cl. perfringens*, *Cl. tetani* and *Staphylococcus aureus*, showed that X-rays caused a partial inactivation of each toxin, tetanus toxin being the most resistant. Loiseleur & Crovisier (1943) were able to increase the effect of X-rays on tetanus toxin by bubbling oxygen through the solution during irradiation.

Most of these investigators, however, were able to obtain only a partial destruction of toxins, and the very wide range and unknown degree of purification of the preparations used in all these works make it quite impossible to draw any conclusions as to the actual sensitivity to X-rays and radium of the toxins used. Moreover, the quantitative results of different experiments cannot be compared and cannot be used as a reliable basis for further investigations. We therefore decided to investigate the conditions favourable for the complete destruction of bacterial toxins by X-rays, the properties of the toxins after irradiation, and the influence of the medium on the sensitivity of toxins to X-rays.

For this purpose two toxins were used, tetanus toxin and *Staph. aureus* haemolysin.

EXPERIMENTAL

Methods

Media used for the production of toxins, and purification of the toxins. Two preparations of tetanus toxin were used.

(1) Crude tetanus toxin. *Cl. tetani* was grown according to Mueller & Miller (1942), and 3% tomato juice was added to the medium. The toxin solution obtained contained 4·5 mg. N/ml., and upon assay on mice was found to contain 1000 minimum lethal doses (Min.L.D.)/ml.

(2) Purified tetanus toxin. This toxin was obtained and purified according to Eaton & Cronau (1938). The purified solution contained 0·14 mg. N and 1000 Min.L.D./ml.

(3) *Staph. aureus* haemolysin. *Staph. aureus* was grown on the medium of Favorite & Haman (1941). The filtrate contained 0·95 mg. N/ml., and up to a dilution of 1 : 200 was capable of haemolyzing rabbit corpuscles. No purification of the haemolysin was attempted. The haemolysin was assayed as follows: 0·5 ml. of serial dilutions of the

toxin were added to 0·5 ml. samples of a 2% suspension of rabbit red-blood corpuscles in saline, and were incubated for 2 hr. at 37°, after which they were refrigerated overnight. The coagulase activity of the same filtrate was tested according to the method of Williams & Harper (1946).

Irradiation. This was carried out with an Ott tube, operated at a tension of 35 kV., and at a current of 5 ma., with a copper anticathode. The distance between the vessel in which the toxin was irradiated and the focal point was 50 mm., and the intensity of the X-rays was about 90,000 r./min. The window consisted of aluminium foil 30 μ. thick.

In the case of tetanus toxin, 0·1 ml. samples of toxin solution containing 100 Min.L.D. were irradiated in cups 15 mm. in diameter. No visible changes were induced in the solutions by irradiation. A control sample (0·1 ml.) containing 100 Min.L.D., kept near the experimental sample during the irradiation, was serially diluted with saline so that 0·5 ml. of the final solution contained 1–50 Min.L.D. After irradiation, the treated sample was diluted serially in an identical manner. At least four white mice, averaging 20 g. in weight, were inoculated intramuscularly with 0·5 ml. of each dilution of the irradiated and the untreated control toxin.

Irradiation of haemolysin was done under the same conditions as for tetanus toxin; 0·1 ml. was irradiated in the same type of cup, and the assays were performed by using identical serial dilutions of irradiated and control samples.

RESULTS

Tetanus toxin. Purified tetanus toxin is destroyed by irradiation much more readily than crude toxin. Comparison of Tables 1 and 2 shows that a dose of 7 million r., under the conditions of the experiment, completely destroyed the purified toxin, whereas the same dose reduced the activity of the crude toxin preparation by only 20%.

X-irradiation destroys the toxicity and the antigenicity of tetanus toxin simultaneously. An attempt was made to immunize mice with purified irradiated tetanus toxin. Purified tetanus toxin was irradiated with 7 million r., thereby completely destroying its toxicity. Twelve mice were intramuscularly inoculated with this irradiated toxin, each mouse receiving four injections, at 2-day intervals, of a dose corresponding to 100 Min.L.D. of the original potency. Three weeks after the last injection the mice were divided into three groups, receiving respectively 2, 5 and 10 Min.L.D. of the original unirradiated toxin. A control group of 12 untreated mice received the same doses at the same time.

All the 24 mice both in the control and the experimental groups died, those receiving 10 Min.L.D. after 3 days, those receiving 5 Min.L.D. after 5 days and those receiving 2 Min.L.D. after 6 days, irrespective of whether they had previously received irradiated toxin or not. This clearly demonstrates that X-ray irradiation destroys the antigenicity of tetanus toxin in the same dose that destroys its toxicity.

Staphylococcus haemolysin. Table 3 shows that, under the conditions of the experiment, 5 million r. completely destroyed the activity of our haemolysin

Table 1. *Irradiation of purified tetanus toxin*

	X-ray doses in millions r.																						
	7			5				4				3				2			Control			
Min.L.D.	100	50	20	100	50	20	10	100	50	20	10	50	20	10	5	20	10	5	20	10	5	1
Days																						
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	T.	T.	.
2	.	.	.	T.	.	.	.	T.	.	.	.	T.	T.	.	.	T.	T.	.	.	+	.	T.
3	.	.	.	+	.	.	.	+	.	.	.	+	T.	.	.	+	.	.	.	.	+	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	0	.	.	+	.	.	.	.	+
5–10	0	0	0	.	0	0	0	.	0	0	0	.	Δ	.	0	.	.	0	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Table 2. *Irradiation of tetanus toxin prepared in a synthetic, protein-free medium*

	X-ray doses in millions r.																							
	7				5					4					3				2			Control		
Min. L.D.	100	50	20	10	100	50	20	10	5	100	50	20	10	5	50	20	10	5	20	10	5	20	10	5	1
Days																									
1	.	T.	.	0	.	.	T.	0	0	.	.	T.	0	0	.	T.	.	0	.	T.	.	.	T.	0	0
2	.	+	T.	.	.	.	.	.	0	.	.	.	T.	.	.	+	T.	.	.	.	T.	.	T.	T.	T.
3	.	.	.	.	.	.	+	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+	T.	.
4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T.	.	.	.	.
5	.	.	T.	0	.	.	.	T.	0	.	.	.	T.	0	.	.	T.	0	.	+	T.	.	.	+	+
6–10	.	.	Δ	.	.	.	.	Δ	.	.	.	.	Δ	.	.	.	Δ	.	.	.	Δ	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

preparation, while smaller doses led to only partial destruction.

Destruction of staphylococcus coagulase by X-rays was tested with the same filtrate that was used as haemolysin. It was found that a dose of 3 million r. completely destroyed the coagulase activity.

Table 3. *Irradiation of staphylococcus haemolysin*

	X-ray doses in millions r.				
Dilutions	5	3	2	1	Control
1/10	0	±	++	++	++
1/20	0	±	+	++	++
1/50	0	0	±	+	+
1/100	0	0	0	±	+
1/200	0	0	0	0	±

++ complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

X-rays simultaneously destroy the toxicity and the antigenicity of staphylococcus haemolysin. A rabbit was intravenously inoculated every 2 days with 0·3 ml. of irradiated haemolysin, receiving a total of twelve injections. A control rabbit received an identical series of injections of unirradiated active haemolysin. Three weeks after the last injection serum from each rabbit was tested for its haemolysin-neutralizing power. As a control we also tested normal rabbit serum. The sera were serially diluted up to 1:2000; 0·1 ml. of haemolysin was added to 0·1 ml. of each dilution.

Table 4. *Action of immune sera (staphylococcus haemolysin) on rabbit corpuscles*

Serum dilutions	Serum of animals immunized with irradiated haemolysin	Serum of animals immunized with active haemolysin	Normal serum control
1/10	0	0	0
1/20	0	0	±
1/50	+	0	+
1/100	++	0	++
1/200	++	0	++
1/500	++	0	++
1/1000	++	0	++
1/2000	++	+	++

++ complete haemolysis; + almost complete haemolysis; ± partial haemolysis; 0 unaffected.

Table 4 shows that both normal serum and serum from the rabbit that had been inoculated with irradiated haemolysin prevented haemolysis up to a dilution of 1 : 20, whereas serum from the rabbit which had been inoculated with active, unirradiated haemolysin prevented haemolysis up to a dilution of 1 : 2000. Thus, irradiation of staphylococcus haemolysin destroyed its antigenicity together with its haemolytic power.

PART 2. THE INFLUENCE OF ACCOMPANYING SUBSTANCES IN THE MEDIUM ON THE SENSITIVITY OF BACTERIAL TOXINS TO X-RAYS

The mechanism of the effect of irradiation on toxins and other biological materials was first studied by investigating the effect of the concentration of the biological substance on the ease with which it was destroyed. It was very quickly observed that the substances in the medium accompanying the active biological material were of the greatest importance in determining the sensitivity of the material to irradiation.

As early as 1909, Courmont & Nogier showed that the inactivation of tetanus toxin by ultraviolet rays proceeded much more rapidly if they diluted the medium with distilled water rather than with bouillon. They explained this effect by assuming that the bouillon contained substances which protected the toxin from the effect of ultraviolet radiation. Friedewald & Anderson (1941), working with Shope papilloma virus, showed that the purification of extraneous proteins from the medium greatly decreased the resistance of the virus to X-rays, whereas the addition of protein to the purified virus solution increased its resistance to X-rays. Dale (1940, 1942) and Tytel & Kersten (1941) found that the sensitivity of enzymes to X-ray irradiation was dependent on the degree of purification of the preparation, and at low concentrations on the concentration of the enzyme in the medium. Similar results were obtained by Goldhaber & Leibowitz (1943) with ultraviolet radiations, and by Luria & Exner (1941) in the action of X-rays on bacteriophage. Evans, Slaughter, Little & Failla (1942) carried out detailed experiments on the influence of foreign proteins and a peptide on the X-ray sensitivity of the fertilizing power of *Arbacia* spermatozoa. Lea, Smith, Holmes & Markham (1944) noted the protective effect of gelatin on the inactivation of tobacco mosaic virus by X-rays.

All these studies make it quite clear that proteins, their degradation products and other organic compounds protect a wide range of biologically active materials from the destruction normally caused by electromagnetic radiations. It seemed interesting, therefore, to determine whether the same effect could be demonstrated with bacterial toxins, and whether any light could be thrown on the mechanism of such an effect.

EXPERIMENTAL

Materials used. Proteins: crystalline egg albumin, bovine serum globulin, casein, gelatin, protamine, edestin.

Peptones: albumin, serum globulin, casein peptones.

Protein hydrolysates were prepared from egg albumin, serum globulin and casein by autoclaving for 10 hr. with

25% (v/v) sulphuric acid. The latter was removed as $BaSO_4$, and the filtrate neutralized with NaOH.

Amino-acids: the mixture of amino-acids used was prepared in the proportions in which they are found in egg albumin (Schmidt, 1938). Separate amino-acids were also tested.

Reducing agents: ascorbic acid, thioglycollic acid and glutathione.

Oxidizing agents: cystine and maleic acid.

Procedure. Each of the above substances was added in varying concentrations to the toxin preparations which were then irradiated with a dose sufficient to destroy the toxin in the absence of any additions. Careful controls were set up to ascertain whether the added substance by itself in any way affected the activity of the toxin, as for example by a change of pH, oxidation, etc. After irradiation the toxin was assayed in order to determine the lowest concentration of the added substance that would protect the toxin from X-ray inactivation, under the conditions of the experiment.

RESULTS

The protective effect of proteins

Tables 5 and 6 show that tetanus toxin was protected by egg albumin in a concentration as low as 0·5%, while haemolysin was protected by a concentration of 2·5%. In the case of serum globulin, a concentration of 2·5% was insufficient to protect tetanus toxin, but haemolysin was protected in a concentration of 1% (see Table 6).

Protamine was tested only on tetanus toxin, and was found to protect it in a concentration as low as 0·5%. Casein, gelatin and edestin were tested up to a concentration of 2% on tetanus toxin and on staphylococcus haemolysin, and were found to have no protective effect whatsoever.

The protective effect of peptones

From Tables 6 and 7 it can be seen that albumin peptone protects tetanus toxin in a concentration of 2%, and haemolysin in a concentration of 5%; that globulin peptone completely protects tetanus toxin and haemolysin in a concentration as low as 2%. Casein peptic, tryptic and papain peptones were all found to have no protective effect in a concentration as high as 5%.

Methylation of the albumin peptone used above with diazomethane did not decrease its protective effect, demonstrating that the free carboxyl groups are not essential for this property.

Comparing the protective effects of proteins and peptones, it is clear that the latter are considerably more effective than their corresponding proteins. This is particularly clear in the case of serum globulin, which does not protect tetanus toxin at all up to a concentration of 2·5%, whereas globulin peptone completely protects it at a concentration of 2%.

The protective effect of amino-acids

In order to determine whether the total molecular structure of the proteins and peptones or the amino-acid building blocks were the agents responsible for the effects studied, acid hydrolysates of proteins, various amino-acid mixtures and single amino-acids were tested.

Table 5. *Effect of egg albumin on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Albumin																								
	10%					5%				2%			1%			0·5%			0·1%			Control			
Min. L.D.	50	10	5	2	1	50	10	5	2	50	10	5	50	10	5	50	10	5	50	10	5	50	10	2	1
Days																									
1	+	+	+	T.	T.	+	T.	T.	0	T.	.	.	T.	.	.	0	.	.	0	0	0	+	+	T.	T.
2	.	.	.	+	.	.	+	T.	.	+	T.	0	T.	T.	.	T.	.	.	.	.	.	.	.	+	.
3	.	.	.	.	+	.	.	+	T.	.	+	0	+	T.	0	T.	T.	.	.	.	.	.	.	.	+
4	.	.	.	.	.	.	.	.	T.	.	.	.	.	.	.	+	T.	.	0	0	0	.	.	.	.
5–10	.	.	.	.	.	.	.	.	Δ	.	.	.	.	Δ	.	.	Δ	.	.	.	.	.	.	.	.

+ death; T. tetanus; Δ recovery; 0 unaffected.

Table 6. *Effect of proteins and peptones on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

	Albumin (%)			Serum globulin (%)			Albumin peptone (%)					Globulin peptone (%)				Control		
Haemolysin dilution	5	4	2·5	4	2·5	1	5	2	1	0·5	0·1	2	1	0·5	0·1	.	.	.
1/10	++	++	0	++	+	±	++	++	++	+	0	++	++	+	±	.	++	.
1/20	++	+	0	+	+	0	++	++	+	±	0	++	+	+	±	.	++	.
1/50	+	0	0	+	±	0	++	+	0	0	0	+	+	0	0	.	++	.
1/100	+	0	0	±	−	0	+	0	0	0	0	±	0	0	0	.	+	.

Table 7. *Effect of peptones on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Albumin peptone (%)																			
	2				1				0·5				0·1				Control			
Min.L.D.	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
Days																				
1	+	T.	T.	0	T.	T.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	T.	T.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	+	.	T.	.	T.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	+
5–10	.	.	.	Δ	.	.	Δ	Δ	.	Δ	0	0	0	0	0	0	.	.	.	.
	Globulin peptone (%)																			
1	+	T.	T.	.	T.	.	.	.	.	.	.	.	.	.	.	.	+	+	T.	.
2	.	+	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	+	T.
3	.	.	+	T.	.	.	T.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	.	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	.	+
5–10	.	.	.	.	.	.	Δ	.	+	0	0	0	0	0	0	0	.	.	.	.

\+ death; T. tetanus; Δ recovery; 0 unaffected.

Acid hydrolysates of egg albumin, serum globulin and casein were studied and were found to be much less effective than the original proteins and peptones. Thus, albumin and globulin hydrolysates showed partial protection of both toxins at 5%, very little at 2%, and none at all at 1%. Casein hydrolysate did not protect at all, up to 5%. An amino-acid mixture, in the proportions present in egg albumin, was approximately as effective as were the acid hydrolysates, and no more. It should be noted that the mixture contained cystine, and not cysteine.

Of all the amino-acids tested singly, only tryptophan, cysteine and methionine were effective as protective agents, while all the others, singly or in groups, were ineffective. Thus, almost complete protection of haemolysin was obtained with cysteine at 0·1%, with tryptophan at 0·5% and with methionine at 2%. Tyrosine could not be tested because at pH 7 it was insoluble in adequate concentrations. Glutathione was an effective protective agent in a concentration of 0·5%. Cystine on the other hand, was totally ineffective.

At this point it became obvious that the effective amino-acids were those that had reducing properties, and additional reducing agents were therefore tested.

Reducing and oxidizing substances

The results with these substances are summarized in Tables 8, 9 and 10. Thioglycollic acid was found to afford protection of haemolysin in a concentration of 0·5% and of tetanus toxin in a concentration of

Table 8. *Effect of reducing substances upon the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

	Cysteine (%)			Cystine* (%)		Glutathione (%)			Thioglycollic acid (%)			Methionine (%)			Tryptophan (%)			Control		
Haemolysin dilutions	1	0·1	0·05	0·5	0·1	0·5	0·1	0·05	0·5	0·1	0·05	2	0·5	0·1	1	0·5	0·1	.	.	.
1/10	++	++	+	±	−	++	+	−	++	++	±	++	++	±	++	++	+	.	++	.
1/20	++	+	±	±	−	±	±	−	++	+	±	+	+	−	+	+	±	.	++	.
1/50	+	+	−	−	−	−	−	−	±	±	−	±	−	−	±	±	−	.	+	.
1/100	±	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−	.	+	.

* Present in the form of a precipitate.

Table 9. *Effect of reducing substances on the inactivation of tetanus toxin by X-rays (irradiated with 7 million r.)*

	Cysteine (1%)				Cysteine (0·5%)				Thioglycollic acid (1%)				Thioglycollic acid (0·5%)				Control			
Min. L.D.	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2	50	10	5	2
Days																				
1	+	.	.	.	T.	.	.	.	+	.	.	.	T.	.	.	.	+	+	.	.
2	.	T.	.	.	.	.	.	.	.	T.	.	.	+	.	.	.	.	.	T.	.
3	.	.	T.	.	+	T.	.	.	.	.	T.	.	.	T.	.	.	.	.	.	T.
4	.	+	.	.	.	.	.	.	.	+	.	.	.	.	.	.	.	.	+	.
5–10	.	.	Δ	0		Δ	0	0	.	.	Δ	0	.	Δ	0	0	.	.	.	+

Table 10. *Effect of ascorbic acid on the inactivation of haemolysin by X-rays (irradiated with 5 million r.)*

Haemolysin dilutions	Ascorbic acid (%) 0·25	0·05	0·01	0·005	Control
1/20	++	+	+	±	++
1/50	+	+	±	−	+
1/100	±	±	−	−	±

1%. Since a number of the effective substances contained SH groups an attempt was made to demonstrate a protective effect with H_2S. This was not possible, however, since H_2S always destroyed the toxin.

As an example of an entirely different type of reducing agent, ascorbic acid was tested and found to protect haemolysin in a concentration of 0·05%. Thus, ascorbic acid was by far the most effective protective agent of all the substances tested. It should be noted that tetanus toxin was destroyed by ascorbic acid, and it could not therefore be tested on this toxin. All attempts to reactivate the X-ray-destroyed haemolysin with ascorbic acid were entirely unsuccessful, thus suggesting that the effect of X-rays on haemolysin cannot be reversed by reduction.

Oxidizing substances such as cystine and maleic acid were totally ineffective as protective agents.

DISCUSSION

The experiments reported above in Part 1 provide an additional example of a phenomenon repeatedly observed with biological materials, namely, that as long as the substance studied is not absolutely pure, it is impossible to draw any conclusions as to its specific sensitivity to X-rays. Thus, partially purified tetanus toxin was found to be far more sensitive to X-irradiation than a crude preparation, 7 million r. destroying 100% of the purified toxin, but only 20% of the crude toxin. Moreover it seems probable that further purification would have increased the sensitivity of the toxin even more. These results are very similar to those of Friedewald & Anderson (1941) working with Shope papilloma virus, of Dale (1940, 1942) and Tytel & Kersten (1941) working with various enzymes and of Luria & Exner (1941) working with bacteriophage, etc.

Comparing the toxins we have studied, it is probable that the haemolysin is more sensitive to X-rays than the tetanus toxin, since the purified tetanus toxin solution containing 0·14 mg. N/ml. was approximately twice as resistant as the crude haemolysin solution containing 0·95 mg. N/ml.

In the case of both of these two toxins, the antigenicity was found to be destroyed simultaneously with the toxicity, thus precluding the use of X-rays for the production of toxoids. This phenomenon is not a general one, as it was found by various authors that toxicity and infectivity were sometimes more easily destroyed by X-rays than were antigenic properties (Friedewald & Anderson, 1941; Moore & Kersten, 1936).

Turning to the mechanism of the action of X-rays on toxins (Part 2), it is now generally accepted that the effect of X-rays on substances in dilute aqueous solution is indirect, the radiation causing ionization and other dissociations of water molecules, which produce OH^- and H^+ ions, H_2O_2 molecules, H and HO_2 radicals and other active groups. The end result will depend on whether the substance in solution is more easily destroyed by oxidation or by reduction (Fricke, 1935; Weiss, 1944).

In the case of simple organic substances it is possible to tell by analysis whether the effect of X-rays has been one of oxidation or reduction. In the case of complex, biologically active materials this is obviously impossible, and it is therefore necessary to resort to the study of the protective effects of added extraneous substances.

In the case of the toxins here employed the conclusion that X-ray destruction in dilute aqueous solution is brought about by oxidation of the toxin fits all the observed facts well. Indeed, the ability of a substance or a mixture to protect the toxin from destruction by X-rays was in direct proportion to the magnitude of its reducing properties.

(1) The only amino-acids that were effective, cysteine, methionine, and tryptophan, were those known to have reducing properties or that could acquire them on irradiation (Mirsky & Anson, 1936). Glutathione evidently acted in the same way.

(2) Reducing substances entirely unassociated with proteins, such as thioglycollic acid and ascorbic acid, were among the most effective protective agents.

(3) Oxidizing substances, such as cystine and maleic acid, had absolutely no effect.

It is very interesting to note that Forssberg (1947), working with solutions of pure catalase, has observed the opposite effect, namely, that the destructive effect of X-rays on catalase in dilute aqueous solution is prevented by oxidizing substances, such as cystine, maleic acid, oxidized glutathione, etc., and enhanced by reducing agents such as cysteine, glutathione, etc. The obvious conclusion is that catalase was sensitive to reduction whereas the toxins used in this study were sensitive to oxidation.

On this basis, it is possible to explain a number of observations that would otherwise be very obscure. The peptones were found to be more effective than the corresponding proteins, since reducing groups are presumably set free during partial hydrolysis. The total acid hydrolysates of the proteins were less effective than the proteins themselves, since tyrosine

and tryptophan are destroyed and cysteine may be partially oxidized during the process.

It is possible to explain the mechanism of the protective effect of proteins in one of several ways: (1) The protein molecules in solution may act by screening the substance in solution from the destructive effect of X-rays, i.e. passive protection; (2) active protection may be induced, whereby the protein molecules actually intervene in the reaction between the active groups produced in the water and the substance in solution, either by combining with the substance and thus stabilizing it (Tytel & Kersten, 1941) or by reacting with the active groups thus inactivating them.

The experiments reported above clearly show that the protective effect of the proteins cannot be explained by the first mechanism, since it is not dependent on the molecular size and shape or on the amount of extraneous materials in the solution. Indeed, a number of proteins, such as casein, gelatin and edestin, and many amino-acids singly or in combination are completely inactive even in very high concentrations, although other proteins, such as egg albumin and serum globulin, and some amino-acids very similar in their general molecular structure to the inactive ones, are very active in low concentrations. It is also extremely improbable that the same toxin can combine with such a wide variety of molecules as were found to be effective protective agents, thus excluding the explanation that the protective agents react with the toxin and stabilize it.

We are thus left with the last possibility, namely, that the protective agents combine with the active groups formed in the water and destroy their activity. The native proteins here tested show no reducing properties, but it is known that denaturation sets free reducing groups, e.g. SH groups (Mirsky & Anson, 1936) and, since X-rays cause denaturation of proteins, it is reasonable to believe that the protective effect of proteins was due to the reducing power of their denatured products, just as in the case of the reducing amino-acids.

Gelatin has only very slight reducing properties, and it was found to have no protective effect on the toxins tested. It is noteworthy that gelatin acts as a very effective protective agent for tobacco mosaic virus against X-rays (Lea *et al.* 1944). The mechanism in that case must be different from the one which is effective for tetanus toxin and staphylococcus haemolysin.

SUMMARY

1. Tetanus toxin and staphylococcus haemolysin were destroyed by X-rays. Purification of tetanus toxin greatly increased its sensitivity to X-rays.

2. In both cases the antigenicity was destroyed simultaneously with the toxicity.

3. Certain proteins, their partial and total hydrolysis products, reducing amino-acids, glutathione, thioglycollic and ascorbic acids were found to be effective as protective agents, whereas oxidizing substances were ineffective.

4. All the experimental observations support the theory that the mechanism of the effect of X-rays on tetanus toxin and staphylococcus haemolysin is an indirect one, the radiation causing the formation of active oxidizing radicals in the water, which in turn destroy the toxin by oxidation.

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The Purification of Antitoxic Plasmas by Enzyme Treatment and Heat Denaturation

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The work of Parfentjev (1936) refocused attention on the possibility of refining antitoxins contained in immune plasmas by the use of enzymes (originally claimed in British Patent No. 18340, Imray, 1902). Later work by Pope (1938), confirmed by the ultracentrifuge studies of Peterman & Pappenheimer (1941), showed that the molecule of diphtheria antitoxic protein may be split by treatment with certain enzyme preparations into two portions of approximately equal size but having different properties, one fragment containing the whole of the antitoxic property of the original molecule. These smaller protein units could still be heat denatured, and Pope (1939*a*) showed that the rate of denaturation of the non-antitoxic portion was very much greater than that of the portion which carried the antitoxic properties of the original unmodified antitoxic molecules. Pope used these findings as the basis of a method for the purification of antitoxic plasmas, consisting essentially of two stages: (1) The antitoxic plasma is treated with pepsin; this splits the antitoxic molecule into two portions, only one of which bears the antitoxic property of the original. (2) This enzymic splitting is followed by critical heat denaturation in the presence of salts and chemical denaturants to precipitate non-antitoxic protein, leaving the modified antitoxic protein in solution.

Pope's original work was carried out on diphtheria and other antitoxin solutions (Pope, 1938) and the results obtained gave information on which a large-scale process could be based. There was, however, little detailed quantitative knowledge relating to the numerous factors which might influence the yield and the degree of purification of the antitoxic protein.

The object of this paper is to describe the large-scale process eventually evolved for refining horse-blood plasmas containing antitoxins and for the concentration and preparation of the refined products for therapeutic use. The process has an intrinsic interest since it involves reactions with labile biological materials of fundamental importance. The development from the original observations which formed the basis of the method to the large-scale process gave rise to numerous problems not encountered in the laboratory. Further problems arose from the increase in the scale of operation which necessitated different ways of handling (involving heating and filtration) during processing. Since some of the problems encountered may arise from fundamental properties of the plasma constituents they should be recorded, as they may suggest new fields for experimental study. Furthermore, the increase in size of the operations magnifies the effect of some plasma constituents which might otherwise be unobserved; processes in which blood plasma is routinely handled in volumes measured in hundreds of litres are not common.

EXPERIMENTAL

Methods and materials

In outline, the purification process comprises two stages: (*a*) pepsin treatment; (*b*) critical denaturation of non-antitoxic protein on samples of antitoxin solution (obtained by the fractional precipitation of antitoxic plasma with $(NH_4)_2SO_4$, followed by dialysis to remove the $(NH_4)_2SO_4$) or antitoxic plasma, varying one or more of the different factors involved in separate experiments. Thus, the method used in each experiment, unless otherwise specified, was as follows:

(*a*) *Pepsin treatment.* Antitoxin solution or antitoxic plasma was diluted with the requisite volume of cold tap water, the dilution adjusted to the required pH with solid citric acid and pepsin powder added. After the action of the pepsin a sample was taken (stage A), the sample being neutralized to *c.* pH 7 with 40% (w/v) NaOH, filtered and the filtrate analyzed for total protein and antitoxin unitage.

(*b*) *Denaturation.* After the action of the pepsin the required quantity of solid $(NH_4)_2SO_4$ was added and dissolved, the pH adjusted as required with 40% (w/v) NaOH or solid citric acid. Portions of 100 ml. were then heated in a water bath at 55° (±0·5°) in 120 ml. rubber-stoppered glass bottles. The duration of this heat denaturation was measured from the time of immersion in the bath. Samples were taken after the addition of the $(NH_4)_2SO_4$ and adjustment to the pH required for heat denaturation (stage B), filtered and the total protein and antitoxin estimated in the filtrates. Finally samples were taken after heat denaturation (stage C) and similarly analyzed.

Total protein. This was estimated by a refractometric method (Pope, 1939*b*).

Antitoxin. Diphtheria antitoxin has been used except where otherwise stated. The antitoxic content was estimated by the flocculation method (accurate to ±5%). From time to time these results have been checked by intracutaneous injection in guinea pigs (Römer & Sames, 1909).

Degree of purification of antitoxin protein. This was evaluated in terms of antitoxic units/g. protein (u./g.); the increase in purity is denoted by *R*, which represents the ratio of the final u./g. to the initial u./g. (Pope, 1939*b*).

Percentage yields. These have been corrected for the dilution involved when solid $(NH_4)_2SO_4$ is added to a given volume of liquid.

Pepsin. Throughout the work pepsin refers to a powder preparation of B.P. 1 : 2500 quality (Chas. Zimmerman and Co. Ltd., London).

The large-scale process

The process finally evolved, using the data obtained from numerous experiments carried out as just described in order to choose the optimal conditions for each operation, can be divided into the following operations:

(1) Bulking of plasma followed by dilution.
(2) Adjustment of pH and addition of pepsin.
(3) Readjustment of pH and addition of $(NH_4)_2SO_4$.
(4) Heat denaturation and filtration.
(5) Recovery of purified antitoxic protein by precipitation with $(NH_4)_2SO_4$.
(6) Dialysis followed by addition of antiseptic.
(7) Ultrafiltration.
(8) Clarification and adjustment of tonicity.
(9) Sterilization.

Experimental details of the above operations are given below.

(1) *Bulking of plasma followed by dilution.* Bulked plasmas of similar antitoxic value (to a total volume of 140 l.) were used whenever possible as this provided a more constant starting material and gave greater flexibility in the final blending for issue for therapeutic use. Since plasma is viscous it is preferable for handling on a large scale that it should flow freely through pipelines and valves. Experimental study of various dilutions both for pepsin treatment and heat denaturation indicated that a dilution of plasma with 2 vol. of water was optimal (protein concentration 2·5–3·0%) although it was feasible to treat with pepsin at a 1 : 2 dilution and then to add a solution of $(NH_4)_2SO_4$ to give a 1 : 3 dilution for the heat denaturation stage. Dilution was carried out with hot water (55°) to give a final temperature in the mixture of 30°.

(2) *Adjustment of* pH *and addition of pepsin.* Experiment showed that the period usually required for considerable enzymic digestion (24 hr. at 37°) was quite unnecessary for the splitting of the antitoxin molecule to the degree needed to allow the differential heat denaturation. Provided the pH was low, either 2 hr. at 20° or 30 min. at 30° produced sufficient disaggregation to prevent subsequent heat denaturation of the resulting modified antitoxic protein. The pH optimum was 3·2; higher pH values produced insufficient disaggregation over a short period, while lower pH values caused destruction of antitoxin. Although in experimental work citric acid was used, to avoid volume changes, it caused unnecessary buffering of the solution and on a large scale HCl, which could be added either after dilution (as 1 : 4 dilution of conc. HCl), or more conveniently during dilution (as 1 : 15 dilution of conc. HCl), was found preferable. The quantity of pepsin required was small as shown in Table 1; 5 g./l. of plasma were sufficient.

Table 1. *The effect of varying the concentration of pepsin*

(Antitoxic plasma containing 0·70% (v/v) 'tricresol'* +ether mixture (i.e. 0·35% 'tricresol') was diluted 1 : 3. Samples taken after 1, 2 and 3 hr. of pepsin treatment at 19° and pH 3·2, were heated for 2 hr. at pH 4·1 in the presence of ammonium sulphate (140 g./l.). Original material (diluted): protein 2·57%; antitoxin 164 u./ml., 6400 u./g.)

Duration of pepsin treatment (hr.)	Pepsin added (g./l. plasma)	Protein present after denaturation (%)	*R* value	Yield of antitoxin (%)
1	1	0·230	4·21	41·1
	2	0·281	4·56	54·3
	5	0·339	4·23	60·9
	10	0·385	4·01	64·9
2	1	0·254	4·29	46·4
	2	0·332	4·14	58·3
	5	0·411	4·03	70·3
	10	0·403	3·95	67·6
3	1	0·352	4·19	62·3
	2	0·405	3·78	64·9
	5	0·442	3·75	70·2
	10	0·433	3·82	70·2

* 'Tricresol' (Schering) is a liquid mixture of the three isomeric cresols, and is usually added to plasma mixed with an equal volume of diethyl ether, to give 0·35% of 'tricresol' in the plasma.

(3) *Readjustment of* pH *and addition of* $(NH_4)_2SO_4$. The quantity of protein rendered insoluble by heat denaturation and therefore the degree of purification were found to depend on both the pH and concentration of $(NH_4)_2SO_4$ (see Figs. 1, 2). The quantity of antitoxin denatured was also dependent on these two factors as shown by the curves for '% yield'. The maximum yield is of primary importance on a large scale (provided an *R* value of at least 3·0 can be obtained), and for this the essential requirements are high pH and low $(NH_4)_2SO_4$ concentration. It was found, however, impossible to obtain efficient filtration if the pH were above 4·3 and the $(NH_4)_2SO_4$ concentration below 130 g./l.; these values were therefore chosen. (The use of salts other than $(NH_4)_2SO_4$ has not given any improvement and has invariably led to slower rate of filtration. Various adsorbents of the kieselguhr type and also activated charcoal have been added during heat denaturation, but in no case has an increase in purity of the antitoxin been observed, nor have filtration rates been substantially improved.)

At this stage of the process 0·1% toluene is added, which later removes lipid material by adsorption on the heat denatured protein, thus preventing its appearance as a precipitate in the final product. Sodium pyrophosphate (0·2%) is also added to raise the pH and to inhibit the formation of coloured compounds from the 'tricresol', previously added (0·35%) to the plasma as a preservative. The first batches of material processed in stainless steel tanks developed a marked red colour due, as was known from earlier work (Pope, unpublished), to the presence of iron which catalyzed the production of quinones from the 'tricresol'; these react with the proteins to give coloured

products. This catalytic action of iron can be inhibited by pyrophosphate (Pope, personal communication), and in all subsequent batches sodium pyrophosphate has been added.

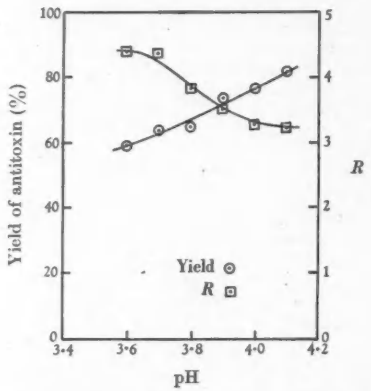

Fig. 1. The effect of pH on the % yield of antitoxin and the degree of purification (*R*). Antitoxic plasma, diluted 1 : 3 and treated with pepsin powder (5 g./l.) for 2 hr. at pH 3·2 and 18°; heated for 2 hr. in the presence of ammonium sulphate (100 g./l.). Original material (diluted): protein 2·32%; antitoxin 156 u./ml.; 6720 u./g.

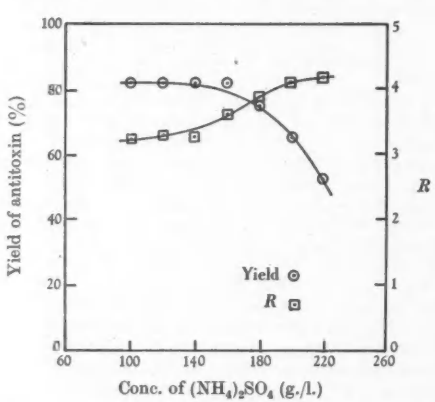

Fig. 2. The effect of ammonium sulphate concentration (g./l.) on the % yield of antitoxin and the degree of purification (*R*) when antitoxic plasma is treated as in Fig. 1, but heating was at pH 4·1.

(4) *Heat denaturation and filtration.* The mixture prepared as just described and adjusted to pH 4·3, is heated in spray-heated stainless steel vessels for 1 hr. at 55°, during which approximately 65% of the original protein is rendered insoluble. It is important that the temperature should be raised to 55° as quickly as possible (20 min.) otherwise peptic action continues, causing the heat-denatured precipitate to become difficult to filter. Heating may be prolonged, leading to increased purity with further slight loss of antitoxin, but higher temperatures, above 60°, markedly increase the rate of destruction of antitoxin.

The concentration of 'tricresol' present during heat denaturation has a marked effect on the purity as shown in Table 2. The concentration of 'tricresol' usually used in these laboratories as a preservative and which gives a concentration of 0·12% on dilution of the plasma 1 : 3 as above is therefore suitable for obtaining a high yield and degree of purification. Many other compounds such as phenol, chloroform, ethanol, acetone and urea were tested in the denaturation stage but none was found superior to 'tricresol'.

Table 2. *The effect of various concentrations of 'tricresol' on the yield and degree of purification of antitoxin*

(Antitoxic plasma diluted 1 : 4 was treated with pepsin (5 g./l.) for 2 hr. at 18°, pH 3·2. Ammonium sulphate (100 g./l.) was then added and the pH adjusted to 4·1. Various percentages of 'tricresol' were then added and the solution heated for 2 hr. Original material (diluted): protein 2·32%; antitoxin 156 u./ml., 6720 u./g.)

'Tricresol' (%)	Protein in filtrate (%)	*R* value	Yield of antitoxin (%)
0·00	0·541	3·24	82·2
0·0125	0·531	3·20	79·4
0·025	0·503	3·38	79·4
0·05	0·442	3·84	79·4
0·10	0·368	4·38	75·2
0·15	0·315	4·83	71·0
0·25	0·252	5·09	59·9

After cooling the liquor to 50° the denatured protein is filtered off in a washing-type ebonite plate and frame filter press and the precipitate washed with $(NH_4)_2SO_4$ solution. By carefully controlling the conditions under which the precipitate is formed it is possible to build up a cake 1 in. thick and still obtain filtration in a reasonable time.

On a large scale an average yield of 65% of the original unitage is obtained in the filtrate plus washings, which are quite clear and colourless.

(5) *Recovery of purified antitoxic protein by precipitation with* $(NH_4)_2SO_4$. The purified antitoxic protein is precipitated from the filtrate obtained as described in section (4) by a further addition of between 160–190 g. $(NH_4)_2SO_4$/l., the quantity added being dependent on the type and potency of the original antitoxic plasma. It is important that the amount of $(NH_4)_2SO_4$ added should be as low as possible in order to minimize concurrent precipitation of proteose, formed in the initial stages of the process by peptic digestion of the serum albumin.

This precipitate of modified antitoxic protein is very difficult to filter and a filter press can only be used with a gravity feed having a head of less than 6 ft. It is then possible to build up a cake 0·5 in. thick. For efficient filtration, the precipitation should be carried out while the filtrate is still warm, but if it is allowed to cool the suspension formed by the addition of $(NH_4)_2SO_4$ may be carefully reheated to 50° before filtering. It is usual to allow this filtration to proceed slowly overnight.

If the filtrate is fractionated by the addition of successive portions of $(NH_4)_2SO_4$ it is found that a first fraction, containing about 10% of the total antitoxin, is of lower purity than the remainder. When processing low-grade antitoxic plasma, it is convenient to add an extra 40 g./l. of $(NH_4)_2SO_4$ to the suspension after heat denaturation in order to precipitate this antitoxin. This is then recovered from the filter press by washing and can be worked up as a recovery batch. If other methods for increasing the degree of purification, such as lowering the pH or increasing the $(NH_4)_2SO_4$ concentration for heat denaturation are adopted the additional lost antitoxin cannot be recovered.

(6) *Dialysis followed by addition of antiseptic.* The cakes of antitoxic protein obtained from the filter press contain a large amount of 30% $(NH_4)_2SO_4$ solution. This is removed by dialysis in cellophan sacs against running tap water, until 0·1% $(NH_4)_2SO_4$ remains; this operation takes approximately 48 hr. After bulking the contents of the sacs 0·35% of 'tricresol' is added as a 10% emulsion in water, which is sprayed into the solution through a perforated stirring tube.

(7) *Ultrafiltration.* It was customary in the early days of this process to press the filter-press cakes of antitoxic protein between sheets of blotting paper in order to remove excess $(NH_4)_2SO_4$ solution before dialysis. It was found however that precipitates of denatured protein sometimes appeared in the final product after candling, possibly due to excess pressure during the pressing. When pressing was discontinued the solution of antitoxic protein obtained after dialysis contained only 9% protein, whereas for therapeutic purposes it is desirable to have the solutions as concentrated as possible, without undue viscosity.

With purified and modified antitoxic protein, 15% solutions are not unduly viscous. The method for raising the concentration to about 15% is ultrafiltration, using collodion membranes deposited on the external surfaces of filter candles. These are immersed in a solution of 8% collodion (Imperial Chemical Industries product) in glacial acetic acid and, after draining, gelled in water. The ultrafiltration takes approximately 7–10 days, but has the merit of removing a portion of the proteose present and also of allowing 'tricresol' to come into equilibrium with the protein. It is carried out by running serum into the stainless steel vessel, which contains five ultrafilters, and applying a vacuum of 29 in. to the bottles connected to the ultrafilters (Fig. 3). Concentrated serum is drawn off from the bottom of the stainless steel vessel every 24 hr.

(8) *Clarification.* It is essential that the ultrafiltered solution should be perfectly clarified to ensure easy filtration through filter candles. This clarification is accomplished by drawing the protein solution through a thick pad of lightly packed paper pulp by means of a low vacuum (5 in. Hg). Autoclaved and finely shredded paper pulp is formed into a pad about 1·5 in. thick in a stainless steel vessel shown in Fig. 4.

(9) *Sterilization.* After clarification, the antitoxin solution is made isotonic by the addition of sterile 20·0% (w/v) NaCl containing 0·1% $NaHCO_3$, in order to bring the solution to pH 6·0, which is the optimum for storage and therapeutic use. The antitoxin solution is then drawn through a filter candle (see Fig. 5) using a vacuum not greater than 20 in. Hg in order to prevent frothing, which has a tendency to cause denaturation of protein. Under these conditions for clarification and sterilization, it is possible to pass 20–30 l. of antitoxin solution (corresponding to two batches) through each pulp and candle.

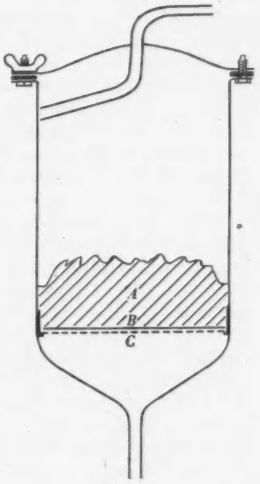

Fig. 4.

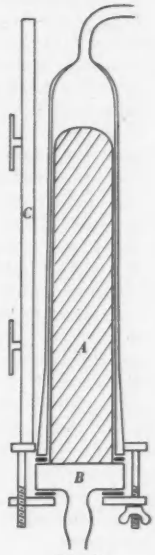

Fig. 5.

Fig. 4. Stainless steel vessel (6 in. diameter) for containing the pad of paper pulp. (*A*) Paper pulp. (*B*) Disk of stainless steel gauze. (*C*) Perforated stainless steel disk.

Fig. 5. Filter candle (10 in.) clamped into glass case (constructed from 2 in. Pyrex pipeline) by means of two stainless steel disks. (*A*) Filter candle. (*B*) Porcelain head of candle. (*C*) Stainless steel supporting bracket.

Applications of the process

Antitoxins. It has been found possible to refine by the process described all true antitoxins, in which the antibody lies largely in the pseudoglobulin fraction of the plasma. A typical series of experimental results is given in Table 3. The *R* values in

Table 3. *Results obtained by processing various types of antitoxic plasma*

(Typical batches of plasma were processed by the method described.)

Type	*R* value	Overall yield of antitoxin (%)
Perfringens antitoxin, type A	3·28	57·4
Septique antitoxin	5·38	57·6
Oedematiens α-antitoxin	4·53	52·5
Tetanus antitoxin	3·84	65·5
Scarlet fever antitoxin	4·97	55·4
Staphylococcus α-antihaemolysin	4·33	66·1
Shiga dysentery neuro-antitoxin	2·99	54·3
Diphtheria antitoxin	3·87	63·2

general vary between 2·5 and 5·0 and are to a large extent dependent on the antitoxic potency of the original plasma, i.e. the higher the antitoxic value of the original plasma, the lower the *R* value. Thus results from 80 batches of diphtheria antitoxic plasma showed a correlation coefficient between *R* value and antitoxic potency of −0·777 which is statistically very significant.

The final product is a perfectly clear straw-coloured liquid, not unduly viscous and showing no signs of precipitation when stored, even at room temperature, over a period of 5 years. It is also remarkably stable, the rate of loss of antitoxic potency at the pH optimum (5·5–6·0) being negligible between 0° and + 5°, approximately 5·0% per annum at 20° and 10·0% per annum at 37° (Harms, 1945). An extremely low incidence of serum reactions has been reported when antitoxic sera refined in the way described have been used therapeutically (Hutchinson, 1939). It has also been shown by Glenny & Llewellyn-Jones (1938) to be absorbed more rapidly and eliminated more slowly than antitoxin purified only by fractional precipitation with ammonium sulphate.

Antibacterial antibodies. Although the fractionation of sera containing antibacterial antibodies with ammonium sulphate has shown that these antibodies are present in the euglobulin fraction, some experiments on refining these sera by the pepsin method were carried out. The results of processing plasma containing antibodies prepared by using as antigen suspensions of the following organisms, *Haemophilus pertussis*, *Proteus vulgaris* OX 19, *Neisseria intracellularis*, and *Erysipelothrix rhusiopathiae* (the organism of swine erysipelas), showed that, as expected, only extremely small antibody yields (less than 20%) could be obtained. Recently a method for concentrating such sera, utilizing the denaturing properties of 'tricresol' has been worked out (Harms, 1946).

The behaviour of separated fractions of antitoxic horse plasma during pepsin treatment and heat denaturation. It has long been customary to concentrate the antitoxic fractions of plasmas by fractional precipitation with ammonium sulphate or other neutral salt, so that the pseudoglobulin fraction, containing the bulk of the antitoxin is partially separated from the fibrin, euglobulin and albumin fractions. Earlier work relating to the process described here was carried out on this pseudoglobulin fraction (Pope, 1939*a*) which was found to behave in a manner similar to whole plasma when processed by the pepsin method. It seemed therefore of interest to investigate the behaviour of the euglobulin and albumin fractions. Very laborious procedure is necessary to obtain electrophoretically homogeneous fractions, but a simple fractionation with ammonium sulphate can give fractions which contain a preponderance of one component. Plasma diluted with an equal volume of water was fractionated by precipitation with ammonium sulphate at the concentration indicated: fraction 1 (euglobulin fraction) 0–220 g./l.; fraction 2 (pseudoglobulin fraction) 220–360 g./l.; fraction 3 (albumin fraction) 360–550 g./l. The three precipitates were then dialyzed.

The solution of each fraction was diluted with tap water to contain 5·0% protein and then an equal volume of 0·24% 'tricresol' in tap water was added to give a concentration of 'tricresol' suitable for heat denaturation and a protein concentration equal to that obtaining when plasma is used. After treatment with pepsin for 1 hr. at 18·5°, ammonium sulphate (130 g./l.) was added and the pH adjusted to 4·25 with 40·0% (w/v) NaOH. Samples (100 ml.) were then heated for 1 hr. at 55°. Protein and antitoxin were estimated on samples taken at stage B (after addition of ammonium sulphate) and stage C (after heat denaturation); total N and non-protein N estimations (Kjeldahl) were carried out on samples at stage A (after pepsin treatment), in order to determine the amount of true digestion which took place. Electrophoretic analyses, for which I am indebted to Mr P. A. Charlwood, were also carried out on the untreated fractions. The results are summarized in Table 4.

Although the fractions into which the plasma had been separated by the ammonium sulphate fractionation were by no means homogeneous, they showed marked differences in composition as determined by the electrophoretic measurements. The results of pepsin treatment followed by heat denaturation also show differences in behaviour. It is clear (Table 4) that the albumin is almost wholly digested while the other proteins show very little digestion. The impure (in terms of u./g. value) fraction 1 gave a degree of purification almost as high as that obtained with fraction 2, due largely to the fact that a very considerable portion of the protein was precipitated by the addition of ammonium sulphate (stage B). It seems logical to suppose that the protein thus precipitated is largely γ-globulin and fibrin (estimated electrophoretically under β-globulin). Most of the antitoxin would appear to be carried by the β-globulin, which apart from being split by enzyme action was largely unaffected by the processing. The figures for fraction 3 however appear to indicate that a part of the β-globulin had no antitoxin associated with it.

The fractions showed marked differences in rate of filtration. Samples from fraction 1 filtered very rapidly and gave brilliantly clear filtrates, whereas with fractions 2 and 3 the filtrates tended to be opalescent. The precipitates formed from fraction 3 were also very sticky and filtered very badly. The good filtration obtained with fraction 1 is in accordance with other observations in which it has been noted that filtration is always better when plasma

Table 4. *The effect of processing fractions of antitoxic horse plasma*

(Antitoxin and protein remaining in solution after processing are given as % of that present in the original fraction.)

Fraction	% of total protein	Protein conc. (%)	Antitoxin u./ml. *In vitro**	Antitoxin u./ml. *In vivo*†	Antitoxin u./g. *In vitro**	Antitoxin u./g. *In vivo*†	Electrophoretic composition (%) Globulin γ	Globulin β	Globulin α	Albumin	Stage in process	Protein (%)	Antitoxin (%) *In vitro**	Antitoxin (%) *In vivo*†	Antitoxin u./g. *In vitro**	Antitoxin u./g. *In vivo*†	Non-protein N/total N (%)
1	33·0	2·52	125	125	4,960	4,960	40·9	38·2	16·3	4·6	A	81·4	—	—	—	—	39·6
											B	36·7	87·0	—	11,600	—	—
											C	23·4	80·8	81·8	17,000	17,200	—
2	47·0	2·55	250	220	9,790	8,620	16·8	43·0	32·9	7·3	A	80·4	—	—	—	—	33·8
											B	70·1	87·1	—	12,000	—	—
											C	37·4	79·7	93·6	20,700	21,400	—
3	20·0	2·54	—	1·5	—	—	0·0	13·5	9·5	77·0	A	31·6‡	—	—	—	—	61·3
											B	25·7	—	—	—	—	—
											C	5·5	—	—	—	—	—

* *In vitro* = measured by the flocculation test.
† *In vivo* = measured by intracutaneous injection into the skin of guinea pigs (Römer & Sames, 1909).
‡ After 2 min. pepsin treatment the protein concentration had diminished to 75·7 % of the original.

is processed rather than serum. There is, therefore, no necessity to convert plasma to serum before processing; in fact it is a disadvantage. It would be an advantage to remove albumin since this would lead to better filtration and obviate the production of proteose and peptone, which tend to contaminate the final product. This, however, would necessitate a laborious initial process involving a difficult filtration.

The treatment of whole blood. An experiment with whole horse blood in comparison with plasma obtained from the same bleeding was carried out. The experiments recorded in Table 5 show that there was a comparatively small increase in total units recovered, while the degree of purification was slightly less when whole blood was used. Concentrates prepared from the filtrates were very much darker in colour; and there would be a danger that breakdown products of haemoglobin might appear in the concentrates. No advantage would therefore appear to be gained by processing whole blood.

Table 5. *The effect of processing whole blood*

(500 ml. of blood were divided into two parts. (A) 250 ml. blood + 750 ml. tap water + 0·12 % 'tricresol', were treated with pepsin (5 g./l.) at pH 3·2 for 2 hr. at 19°. Heat denaturation for 1 hr. at 55°, pH 4·1. (B) 250 ml. of blood were allowed to settle for 24 hr. and the supernatant plasma siphoned off. 184 ml. of plasma were thus obtained and after dilution with 2 vol. of tap water, 0·12 % 'tricresol' was added and the solution treated as (A). The cells and their suspending plasma, after withdrawal of the supernatant plasma, were diluted to 250 ml. with tap water, 0·12 % 'tricresol' was added and the solution was treated as (A). Antitoxin was recovered as recorded below. Original material: blood (diluted): protein 3·15 %, antitoxin 204 u./ml.; 6500 u./g. Plasma (diluted): protein 2·64 %, antitoxin 320 u./ml.; 12,100 u./g. Total antitoxin units in 250 ml. blood = 204,000.)

Material	Units recovered: Total	% of original*	u./g.
Blood	89,225	43·7	40,000
Plasma	82,325	40·4	42,500
Cells	5,719†	2·8	45,400

* Low yields due to mechanical loss in processing small quantities.
† Presumably from the plasma left with the cells.

DISCUSSION

The results obtained both in the laboratory and in large-scale processing show that, in general, disaggregation of a major portion of the antitoxic protein molecules, as judged by the resistance of the antitoxic portion to heat denaturation, takes place very rapidly, even in the presence of very small quantities of pepsin. The destruction of antitoxin which may vary from 20 to as much as 50 % in some batches (even under optimal conditions) may be explained partly by protein denaturation and partly by the resistance of a portion of the antitoxic protein to disaggregation.

When antitoxic sera (including those obtained by the process described herein) containing phenolic preservatives are stored for a long period a small loss in antitoxic potency occurs; this loss is greatly accelerated by rise in temperature and by alterations of pH from the optimum (pH 5·5–6·0). This loss is caused by denaturation of the antitoxic protein (Harms, 1945) and it is probable that a similar process operates during pepsin treatment at pH 3·2 to account for the 10 % loss in antitoxic units which invariably occurs at this stage. Further loss, probably due to the same cause, occurs during the heat treatment, since this loss may be increased by raising the temperature or increasing the time of heating. Whereas the loss of antitoxic units during pepsin treatment is constant, that occurring during the succeeding stages (B and C) of the process is very

variable and cannot be accounted for by heat denaturation of disaggregated antitoxic protein. Thus, when ammonium sulphate is added at stage B a quantity of antitoxic protein, which varies considerably from batch to batch, is precipitated. Should the quantity of ammonium sulphate added be lowered in order to decrease this precipitation it is found that an extra loss equivalent to this increase occurs during stage C. The most probable explanation of this phenomenon is that the globulin which can be precipitated by a low concentration of ammonium sulphate carries an antitoxic protein resistant to disaggregation by pepsin treatment. This is confirmed by the observation that when antitoxic globulin solutions prepared by the ammonium sulphate fractionation of immune plasma (during which a fraction of the globulin, insoluble in low concentrations of ammonium sulphate, is removed) are processed, yields are generally higher than when immune plasmas are processed.

Rosenheim (1937) found that whereas *Eberthella typhosa O* agglutinins were destroyed by pepsin treatment, *H* agglutinins were more resistant and the resistance depended on the degree of immunization of the horse from which they were obtained. Also it has been found that antibacterial antibodies are almost completely destroyed when processed by the method described in this paper. The work of Cohn, Oncley, Strong, Hughes & Armstrong (1944) on the ethanol fractionation of human plasma indicates that many of the antibodies, including antitoxins, are associated with the γ-globulin fraction. It is possible that the method of immunization of horses affects the distribution of antitoxin between the β-globulin and γ-globulin fractions. If, as evidence would seem to indicate, the γ-globulin of antitoxic horse plasma is more resistant to disaggregation by pepsin than the β-globulin, this may account for the destruction of antibacterial antibodies because these are largely confined to the γ-fraction and it might account for the variable yields obtained with different batches of antitoxin. The experiment (Table 4) in which three fractions of antitoxic horse plasma were processed shows that very much more protein was precipitated and heat-denatured in fraction 1, containing the high proportion of γ-globulin, than in fraction 2 which contained a much smaller proportion. Although the yield of antitoxin from both fractions was substantially the same, this can be explained by the fact that fraction 1 still contained a high percentage of β-globulin, with which the majority of the antitoxin was associated.

Further work on pure globulin fractions is indicated in order to clarify this point, for it has been suggested that the loss of certain portions of the antitoxic protein during purification may have led to the failure of antitoxin therapy in some types of diphtheria infection (O'Meara, 1939).

The purity of antitoxin (u./g.) which can be obtained depends mainly on the purity of the original plasma, although the presence of antitoxins other than the specific one, formed either during the course of immunization or during a previous immunization to produce another type of antitoxin, will contribute protein to the filtrate after heat denaturation and so lower the final purity.

Should it be possible to produce an immunization in a horse in which only one specific antitoxin was formed and the horse possessed no significant quantity of other antitoxin, it seems possible that 'pure' antitoxin could be produced by the pepsin process. Using diphtheria antitoxic plasma containing 3600 units of antitoxin/ml. it was possible to obtain an antitoxin solution of 100,000 u./g., which is of the same order as that produced from refined antitoxin by the method of Pope & Healey (1939) using toxin-antitoxin floccules. Experimental evidence indicates that, even under optimal conditions, the degree of purification and yield of antitoxin which can be obtained by pepsin treatment followed by heat denaturation are limited by the quality of the original plasma. Improvement may therefore come from better methods of immunization designed to produce only the specific antitoxin in a form in which it is easily disaggregated by pepsin.

SUMMARY

1. A description is given of a large scale process suitable for the purification of true antitoxins, but not for antibacterial antibodies, by treatment of immune plasmas with pepsin followed by critical heat denaturation in the presence of a protein denaturant. The preparation of the purified antitoxin for therapeutic use is described.

2. It has been established that the optimal conditions on the large scale are pepsin treatment (0·5 hr. at 30° at pH 3·2 with plasma diluted 1 : 3) and heat denaturation (1 hr. at 55° at pH 4·3 in the presence of 130 g. ammonium sulphate/l. and 0·12 % 'tricresol').

3. It has been shown that it is preferable to process plasma rather than whole blood, serum, or salted-out fractions of plasma.

4. The limitations of the process and the bearing of methods of immunization on yields and purity (u./g.) are discussed.

I am indebted to Dr J. W. Trevan and Dr C. G. Pope for their help and advice both during the period in which the work was carried out and in the preparation of the paper; also to Mr A. T. Glenny and his staff who carried out many of the tests for antitoxic potency. I am very grateful to Dr B. C. J. G. Knight for his criticism and help in the preparation of the manuscript for publication.

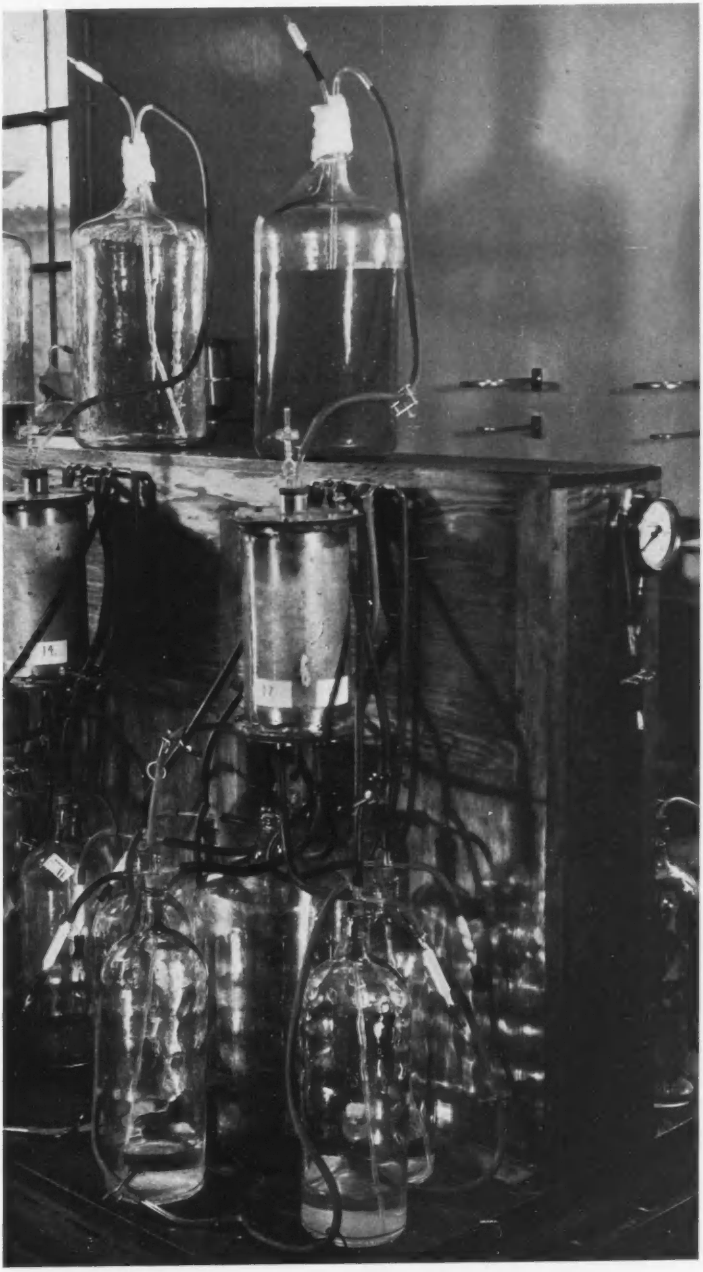

Fig. 3. Photograph showing the ultrafiltration in progress.

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Fructose and Glucose in the Blood of the Foetal Sheep

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Little is known of the distribution of D-fructose in animal tissues, apart from its demonstration in certain embryonic fluids (see, e.g., Needham, 1931, 1942). Recently it has been positively identified by Mann (1946) as the reducing sugar in the seminal plasma; the fact that hitherto it had been taken for granted that this was glucose suggests that fructose may have escaped detection in other instances.

There is no excuse for this, for a characteristic, though not entirely specific, colour reaction, due to Seliwanoff (1887), has been available, and in the methylphenylosazone Neuberg (1902; Neuberg & Strauss, 1902) found a derivative by which it could be distinguished from accompanying glucose, provided always that a sufficiently concentrated solution was available. Our own attention was drawn to the problem of identifying fructose by the late Sir Joseph Barcroft and Mr S. W. Cole, who in the course of recent work on the foetus of the sheep had confirmed an observation of Paton, Watson & Kerr (1907). The latter authors detected by the Seliwanoff test a fructose-like substance in the amniotic and allantoic fluids of sheep, cow, pig, cat, dog, rabbit, ferret and guinea pig, and in foetal blood. They investigated the blood more closely: 'by extracting large quantities of foetal blood [of the sheep?] with alcohol, evaporating with charcoal, and taking up in a small quantity of water, we were able to extract a sugar which was laevorotatory and which gave Seliwanoff's reaction distinctly...'. Orr (1924) obtained similar results using lead acetate instead of charcoal, with blood from a newly born kid, and blood taken from the human placenta after birth.

The observations of Cole & Hitchcock (1946) indicated that appreciable quantities of fructose (up to 100 mg./100 ml.) were present in the blood of the foetal sheep, and at Sir Joseph Barcroft's request we attempted (*a*) to prove that the substance responsible for the colour reaction was in fact fructose, and (*b*) to isolate a derivative of the sugar.

EXPERIMENTAL

Methods

Standard fructose and glucose solutions. These were made in saturated benzoic acid solution (Folin, 1929); a concentrated solution (*c.* 2%) was made for polarimetric measurement of the concentration, and this was diluted with more saturated benzoic acid solution to give a final sugar concentration of 0·5 mg./ml. In our experience a solution of either sugar at this, or a lower concentration, does not deteriorate when kept for several years at room temperature in a well-stoppered bottle.

Total reducing substances (t.r.s.). (More strictly, reducing substances estimated by the ferricyanide-cerium method.) The procedure used was that of Miller & Van Slyke (1936), the solutions being heated in boiling tubes without stoppers, and titrated to the nearest drop (0·02 ml.) after cooling and acidification, using xylene cyanole FF as an internal indicator. Instead of standardizing the ceric sulphate solution and using a fixed time of heating, a standard fructose, and occasionally a glucose solution, containing 100 μg. of sugar, was included in each set of estimations, and heating was carried out for 10–12 min. in a boiling water bath. The ceric sulphate solution was of such a concentration that 1 ml. was equivalent to about 100 μg. of reducing substance, calculated as hexose. Contrary to the findings of Miller & Van Slyke (1936) we found no discrepancy greater than the experimental error, between the reducing values of glucose and fructose, nor did we find any appreciable variability in the reducing value of fructose.

Fructose. For the estimation of those substances giving the Seliwanoff reaction, called here 'fructose' for the sake of brevity, a modification of Roe's method (1934) was used. This was devised by Mr S. W. Cole, who very kindly gave

us the details and provided the facilities for carrying it out. A full account of the application of the method to the estimation of fructose and inulin in blood and urine will be published at a later date.

To the test material contained in 4 ml. of aqueous solution (in the case of the blood filtrates, diluted 1 in 10, 1 ml. plus 3 ml. of water was usually sufficient), in glass-stoppered test tubes, were added first 6 ml. of resorcinol solution (A.R., 0·15% (w/v) in absolute ethanol), thereafter at 1 min. intervals 6 ml. of conc. HCl containing 7·5 mg. $FeCl_3$/l. After thorough mixing of the contents, the tubes were placed at 1 min. intervals in a thermostatically controlled water bath at 75°, and left for 30 min. They were then cooled in jars of cold water, and the colour developed read with suitable filters in 2 cm. cells in a Spekker photo-electric absorptiometer, against a 'blank' on the reagents plus water. Standard fructose solutions containing 50 or 100 μg. were included in each set of estimations; these gave densities of about 0·32 and 0·65 respectively. The majority of readings were made with a spectrum blue filter (Ilford Ltd., no. 602), but in some experiments a Wratten no. 75 (Kodak Ltd.), which gave rather smaller densities, was used instead. (In a very few experiments the bath temperature was 80° and the time of heating then either 20 or 30 min.) Mr Cole tells us that under these conditions the specificity of the method is such that glucose gives between 0·6 and 0·8% of the colour given by an equal amount of fructose, and that the relationship of density to concentration over the range 20–100 μg. is for all practical purposes a straight line.

Yeast fermentation. This was carried out with a commercial (Distillers Company Ltd.) baker's yeast. A sample of the pressed yeast was suspended in ten times its weight of tap water, left for an hour or more at room temperature, centrifuged, and resuspended in water. In some experiments the washing was repeated; in others the yeast was resuspended in phosphate buffer at pH 6. An equal volume of the suspension was added to the test solution and left, with occasional mixing, for three or more hours at room temperature, or for a shorter period at 25°. Under these conditions, as the results below show, fructose is completely removed from the solution.

Estimation of glucose by the use of glucose oxidase. For these estimations we are greatly indebted to Dr T. Mann (cf. Mann, 1946) who provided a solution of the enzyme and assisted in the carrying out of the experiments. The oxidation was allowed to proceed at room temperature in the presence of a trace of catalase, after which appropriate estimations were made, usually without preliminary deproteinization.

Source and collection of blood

All the foetal blood used was obtained for us by Sir Joseph Barcroft, and was collected from a cannula in the carotid artery of the foetus, or, occasionally, by a syringe from the umbilical vessels, when the maximum quantity was wanted. The foetus was in every case still attached by the placenta to its mother who was anaesthetized with spinal Duracaine before the operation, and both were partly immersed in a warm bath. Observations had been made on the foetus and mother during the 1–1·5 hr. between the opening of the uterus and the taking of blood, but these were not considered to have disturbed the metabolism of the foetus unduly. It is likely, however, that the blood sugar level of the mother was in these circumstances higher than before anaesthetization (Hitchcock, 1948), and the glucose level in some of the foetal blood samples may have been raised correspondingly.

The blood was allowed to run through a rubber tube into a convenient volume of distilled water (see below) contained in a stoppered measuring cylinder which had been chilled on ice, the time for collection, even of large samples, not exceeding 5 min. In this way glycolysis was reduced to a minimum. The partly haemolyzed blood was mixed thoroughly, the increase in volume noted, and samples withdrawn for cadmium deproteinization (see below). The amount of blood collected was inferred from the increase in volume. Immediately after the withdrawal of samples more water and 97% ethanol were added, and the haemolysis thereby completed. The mixture was then transferred to bottles of litre capacity stoppered by rubber bungs, and kept cold until the next step in solvent deproteinization.

Deproteinization

With cadmium hydroxide. This was used for the samples of diluted blood, which were used to measure the t.r.s. and fructose contents of the blood, and as a check on the recovery of the sugars in the bulk deproteinization by organic solvents. The method used was essentially that of Fujita & Iwatake (1931). The precipitated material was removed by centrifuging because of the possibility that heating in alkaline solution might convert glucose to fructose. The supernatant fluids obtained were water-clear; for convenience they are referred to as 'cadmium filtrates'.

With ethanol-chloroform. This method is an adaptation by Keilin & Mann (1940) of that used by Tsuchihashi (1923), and we are grateful to Dr Mann for the guidance which he gave us in its use. A typical treatment was as follows: 160 ml. of blood were collected into 200 ml. of water. After withdrawal of two 1 ml. samples for cadmium deproteinization, haemolysis was completed by the addition of a further 250 ml. of water, and 300 ml. of 97% ethanol. To the haemolyzed mixture 150 ml. of chloroform were added and the mixture shaken *very gently*. The resulting precipitate did not flocculate completely. 2 ml. of approx. 20% acetic acid were added to make the suspension orange to methyl red, and after very gentle agitation the precipitate separated rapidly, leaving a water-clear supernatant fluid. The material was left in the refrigerator for 2 hr. and centrifuged. (Centrifuging at this stage gives three layers, an upper aqueous one, an intermediate layer of coagulated protein, and a lower layer of chloroform. When the aqueous layer is decanted the chloroform sometimes runs out as well, but can easily be separated from it.) 755 ml. of supernatant fluid were decanted through glass wool. The protein and chloroform layers were shaken up vigorously with 300 ml. of an emulsion made by shaking a 2 : 1 water-ethanol mixture with an excess of chloroform. The suspension was again centrifuged and 300 ml. of water-clear supernatant fluid decanted through glass wool into the main bulk. The colourless solution was stored in the refrigerator.

In our experience it was necessary to adjust the pH carefully, and not to shake too vigorously at any stage, otherwise the coagulated protein formed a fine suspension which made opalescence in the aqueous phase difficult to see. Ideally, a water-clear fluid should be obtained on centrifuging; slight opalescence, however, did not appear to

interfere with subsequent operations. For convenience, the deproteinized solutions obtained by this procedure will be referred to as 'ethanol-chloroform filtrates'.

Concentration of ethanol-chloroform filtrates. This was done under reduced pressure with a bath temperature not exceeding 50°. When the volume had been reduced to about 20 ml., representing usually a fivefold concentration of the original blood, solid separated, but filtration in the presence of kieselguhr or Hyflo Supercel readily gave a clear pale-yellow solution. Alternatively the last stages of evaporation were done in the presence of a relatively large volume of Hyflo Supercel (e.g. 2 g.) and the blood concentrate was thereby obtained in a dry finely divided form. In a few experiments the concentrate was taken to complete dryness and extracted with absolute ethanol (see below). (All these are called 'ethanol-chloroform concentrates'.)

Recoveries

Fructose. A preliminary experiment showed that fructose added to maternal blood could be recovered in good yield in the ethanol-chloroform filtrate. The comparison of fructose contents of ethanol-chloroform filtrates and cadmium filtrates prepared from the same samples of foetal blood showed that the recovery at this stage was nearly complete (Table 1).

T.r.s. Recovery of t.r.s., though less significant, indicates no great discrepancy between the two methods of deproteinization (Table 1).

The reducing substances in cadmium filtrates of triplicate diluted samples of blood 19 were analyzed for us by Mr M. W. S. Hitchcock, who used the copper-reduction method of Somogyi (1945). Corrected for dilution, these gave an average of 133·3 mg. reducing substances/100 ml. of blood, as against our value of 130·4 mg./100 ml. by the ferricyanide-cerium method.

Ethanol extraction. Table 2 shows the percentage recoveries (in all fractions) of fructose and t.r.s. at several stages of the working up of a sample of the pooled blood filtrates 3, 4, 5, 6. The losses on removal of the ethanol and chloroform are probably mainly fictitious, since these solvents increase slightly the values obtained by both methods (e.g. in the fructose estimation the addition of 1 ml. of the clear supernatant fluid from shaking together 10 ml. water, 5 ml. 97% ethanol, and 2 ml. chloroform increased the 'fructose' value of 50 μg. fructose to 52·4 μg., and of 100 μg. to 103·2).

From these results it was concluded that the ethanol-chloroform filtrates could be taken as reasonably representative of the original bloods, in so far as fructose and t.r.s. were concerned.

RESULTS

Composition of the original foetal blood samples as measured on the cadmium filtrates

The figures in Table 3 give the age of the foetus, and the fructose and t.r.s. contents of the blood sample, measured on the cadmium filtrate (cf. Cole & Hitchcock, 1946). In some cases the amount of blood used was not the whole of that obtained from the foetus. It will be seen that variation in the fructose and t.r.s. contents is apparently at random, although Hitchcock (1948), who took samples from younger foetuses, suggests that the fructose content falls towards the end of term (147 ± 3 days), while the t.r.s.-fructose (which he assumes to be glucose) rises.

Table 1. *Total reducing substances (t.r.s.) and fructose (F) in ethanol-chloroform and cadmium filtrates compared*

Blood sample ...	Amount in sample (mg.)							
	3, 4, 5, 6*		3, 4, 5, 6, 7*		11		19	
	T.r.s.	F	T.r.s.	F	T.r.s.	F	T.r.s.	F
Cadmium filtrate	147	30	105	56	326	158	196	124
Ethanol-chloroform filtrate (evaporated to small bulk)	157	28	98	52	311	137	201	104

* Pooled ethanol-chloroform filtrates from several blood samples; the figures refer to a portion of the combined filtrates. *F* = fructose.

Table 2. *Recovery of fructose and t.r.s. at successive stages of concentration and ethanol extraction of an ethanol-chloroform filtrate*

Stage	Recovery of fructose (%)		Recovery of t.r.s. (%)	
	Between stages	Overall	Between stages	Overall
Initial ethanol-chloroform filtrate	—	(100)	—	(100)
Evaporated to dryness *in vacuo* below 50°	94·8	94·8	88·3	88·3
Extracted with boiling absolute ethanol	86·2	81·8	97·3*	86·0
Ethanolic extract evaporated to dryness *in vacuo*	97·7	80·0	89·9	77·3

* Part in extract, part in residue.

Table 3. *Composition and other data on blood samples, measured on cadmium filtrates*

No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)	No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)
1	107	142	157	70	11	142	71	145	224
2	112	97	—	60	12		75	155	300
3	120	123	142	62	13	141	51	83	184
4*	108	53	84	69	14†	142	56	114	142
5	129	34	61	71	15 †		76	150	180
6		25	57	85	16	141	69	107	160
7	132	69	159	178	17†	141	69	109	250
8†	139	66	—	110	18	124	—	—	50
9†		81	—	178	19	141	82	130	150
10	144	35	86	—	20	—	55	82	—

* Twin died, not sampled. — Not estimated.
† 10–70 ml. of blood taken for other purposes. The bracketed foetuses are twins.

Analysis of concentrates of ethanol-chloroform filtrates

(*a*) *Optical rotation.* This was first measured on an aqueous solution of the absolute ethanol-soluble fraction of the material in an ethanol-chloroform filtrate. In a 2 dm. tube the rotation was −0·73°; that calculated from its fructose content was −0·52°, suggesting that other laevorotatory substances were present, and that they were ethanol-soluble. Subsequently, rotations were read of several ethanol-chloroform concentrates; these are summarized in Table 4 with the values calculated from the fructose contents.

Table 4. *Rotations of concentrates from ethanol-chloroform filtrates compared with the rotations calculated from their fructose contents*

Blood sample ...	3, 4, 5, 6, 7	11	19	12, 14, 16, 37
	(α_D)	(α_D)	(α_D)	(α_D)
Rotation in 2 dm. tube	−0·58°	−1·54°	−1·40°	−2·10°
Rotation calculated from fructose content	−0·32°	−1·00°	−0·75°	−1·42°

These results indicate clearly the presence of laevorotatory material which is not fructose, and illustrate the danger of assuming that laevorotation in itself is evidence for the presence of fructose (cf. Orr, 1924).

(*b*) *Change in optical rotation on treatment with yeast.* If the other laevorotatory material were not yeast-fermentable it should be possible to show that the change in rotation accompanying fermentation is equivalent to the fructose disappearing. To this end a series of experiments was performed. It was essential to begin with a relatively large optical rotation, and fortunately the concentrates of ethanol-chloroform filtrates were almost colourless, and could be read after filtration without the need for decolorization.

In most experiments 6·0 ml. of the concentrate were mixed with 5·0 ml. of a yeast suspension prepared as described above, and the fermentation was allowed to go to completion. The yeast was then centrifuged out, leaving sufficient clear supernatant fluid for the rotation to be read in a 2 dm. tube (capacity *c.* 9·5 ml.). Estimations of fructose were done on the solution before and after fermentation, and the rotation due to the fructose fermented was calculated. When these experiments and those with glucose oxidase indicated the possible presence of glucose, fermentable t.r.s. were estimated also, and the difference between this result and the fructose content taken as glucose; the change in rotation expected on its disappearance was calculated and subtracted from that for the fructose. The results are summarized in Table 5.

These results make it clear that non-fermentable laevorotatory material is present. They also show fairly good agreement between the observed change in rotation and that calculated on the assumption that the fermentable reducing substance is a mixture of fructose and glucose. It will be noticed that in the absolute ethanol-soluble fraction the amount of non-fructose fermentable material is smaller, probably because of the limited solubility of glucose in that solvent.

Table 5. *Effect of yeast treatment on the optical rotation of ethanol-chloroform concentrates: comparison of the observed change in rotation with that calculated from the fructose and t.r.s. disappearing*

		Losses on fermentation				Optical rotation		Change	
		Fructose		Non-fructose					
Sample	Vol. (ml.)	(mg.)	(α_D)	(mg.)	(α_D)	Before	After	Observed	Calculated
11*	25	134·6	−1·00°	115·6	+0·49°	−1·54°	−0·97°	+0·57°	+0·51°
19	25	100·2	−0·75°	13·0	+0·06°	−1·40°	−0·67°	+0·73°	+0·69°
Control	10	120·1	−1·48°	58·6	+0·41°	−1·11°	0·0°	+1·11°	+1·07°
Ethanol extract 11†	25	112·0	−0·82°	15·4	+0·07°	−0·98°	−0·31°	+0·67°	+0·75°

* 10 ml. of the concentrate used for this experiment were evaporated to dryness *in vacuo* below 50°, pH 5, in the presence of 0·5 g. barium acetate and a little acetic acid. The residue, dried exhaustively over H_2SO_4, was extracted with a total of about 100 ml. absolute ethanol, the extract being filtered through Hyflo Supercel on a G4 filter and evaporated to dryness, with the same precautions as to temperature. The residue was extracted with exactly 14 ml. of approx. 2% Na_2SO_4, the $BaSO_4$ being removed by centrifuging. The clear supernatant liquid was used for the second experiment (†).

(*c*) *The action of glucose oxidase on concentrates.* Positive evidence for the presence of glucose was obtained through experiments with glucose oxidase.

A mixture of five ethanol-chloroform filtrates (500 ml.) (3, 4, 5, 6, 7) was evaporated to small bulk under diminished pressure with a bath temperature never higher than 52°. The pH was checked by means of methyl red throughout the concentration, which took 5 hr. A little kieselguhr was added and the suspension filtered by pressure on a G3 sintered-glass filter. The volume of filtrate was made to 30 ml. Samples (2·0 ml.) of this were taken for the estimation of yeast-fermentable t.r.s. and fructose. Other 2·0 ml. samples were taken to study the effect of glucose oxidase (plus catalase) on the t.r.s. and fructose. 200 μg. of enzyme were used in a total volume of 2·9 ml., which included 0·5 ml. of M-acetate buffer (pH 5·2). The solutions were shaken for 1·5 hr. in the cups of Barcroft manometers. Estimations of t.r.s. and fructose were done without deproteinization. A control on the enzyme and buffer was not distinguishable from the blank, and the buffer had no effect on the results of either estimation when added to 100 μg. of fructose. The significant findings are summarized in Table 6.

The agreement between (3) and (4) suggests that all the fermentable reducing substances can be accounted for by fructose and glucose.

A repetition of this experiment with blood sample 19, in which all the solutions were deproteinized before estimations were done, gave rather less satisfactory agreement; the ratio of fructose to glucose in this sample was much higher, about 8 : 1.

(*d*) *Other constituents of the concentrates.* The concentrate used for the experiment of Table 6 contained 82 mg. of lactic acid in 30 ml., corresponding to 91 ml. of mixed blood. (This is not enough to account, as L-lactate, for the non-fermentable laevorotatory material; see (*b*) above, cf. Bell, 1932.) In addition the concentrates gave a strong nitroprusside reaction which was removed by cadmium deproteinization. However, there is evidence that this treatment does not remove all the non-fermentable reducing material (see below). It was thought that extraction with absolute ethanol in the presence of barium acetate might leave behind some of these substances, yet the extract so obtained from

Table 6. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a pooled ethanol-chloroform concentrate*

(All results expressed as mg./30 ml. of the original concentrate.)

	Untreated concentrate		After glucose oxidase treatment		After yeast treatment
	Alone	With buffer	No addition	2 mg. glucose added	
Fructose	50·9	51·6	51·0	50·3	1·2
T.r.s.	97·3	98·6	73·7	73·0	22·3

These results demonstrate that the enzyme had no effect on fructose, and that it had worked to completion. From them were calculated:

		(mg.)
(1)	Fermentable reducing substances	(97·3–22·3) = 75·0
(2)	Fermentable fructose	(50·9–1·2) = 49·7
(3)	Fermentable non-fructose reducing substances	(75·0–49·7) = 25·3
(4)	Glucose from the effect of glucose oxidase	(98·6–73·7) = 24·9

blood sample 11 still contained 50 mg. of non-fermentable t.r.s. ('fructose' content, 4·2 mg.) as against 56·5 before this treatment. The fermented extract still had a strong negative rotation (see Table 5).

We are indebted to others for certain additional information: Miss V. Moyle kindly estimated allantoin for us, in blood concentrate 19, by the method of Young, MacPherson, Wentworth & Hawkins (1944), and found an

amount corresponding to 0·3 mg./100 ml. of the original blood (cf. 1·9 mg./100 ml. in adult sheep's blood, and similar values for cattle and pigs; Fosse, Brunel & Thomas, 1931). Dr S. M. Partridge (Partridge, 1948) has shown that sample 18, and pooled samples (3, 4, 5, 6, 7, 13, 15) and (8, 9, 10) contain inositol. Inositol gives no reduction under the conditions of the ferricyanide-cerium method for t.r.s.

Isolation of fructose derivatives

Methylphenylosazone. A preliminary experiment on maternal blood with added fructose showed that the osazone could be prepared from ethanol-soluble material in an ethanol-chloroform filtrate. This procedure was applied to the first sample of foetal blood.

The ethanol-soluble fraction (0·6 g.) of the material in the ethanol-chloroform filtrate, estimated to contain 57 mg. of fructose, was dissolved in 2·5 ml. of water, in which it was completely soluble, 1 ml. of freshly distilled methylphenylhydrazine added, with 2·2 ml. of 96% ethanol to render the mixture homogeneous, and finally 1 ml. of 50% acetic acid. The darkening solution was warmed in a water bath for 10 min. and left at room temperature for 1·5 hr. 10 ml. of N-acetic acid were then added, and the osazone extracted with successive portions of 20, 15 and 15 ml. of chloroform. The combined extracts were shaken with 25 ml. of N-acetic acid, then with dilute $NaHCO_3$ solution until no more CO_2 was given off, and finally with water. After being dried over anhydrous Na_2SO_4, the chloroform solution was boiled with a little charcoal, filtered, and evaporated to dryness under reduced pressure. The 0·8 g. of residue (*N*-methylaniline was present) was recrystallized from a mixture of chloroform-ligroin (b.p. 40–60°). The melting point was raised to 151–152° by recrystallization from 20% ethanol-water. Yield 37·8 mg. (31% of theory). Under the same conditions 134·5 mg. of pure D-fructose gave 84·6 mg. of osazone (29% of theory). 170 mg. of glucose gave no crystallizable product.

The tetraacetyl derivative was prepared from the osazone from foetal blood: 23·2 mg. were dissolved in 2 ml. of 2 : 1 pyridine-acetic anhydride mixture and left 20 hr. at 40°. 20 ml. of water were added, the mixture shaken vigorously, left 4–5 hr. and filtered. The thoroughly washed material was dissolved in the minimum of hot ethanol, and water was added at boiling point until crystallization began. Yield 27·9 mg. of lemon yellow crystals; m.p. 125–126° (cf. Percival & Percival, 1937).

The yields of the methylphenylosazone are much lower than those claimed by Neuberg in his earlier papers. The highest which we have obtained by following his methods exactly are about 50% of theoretical. We have not applied the new modification of the procedure which he published after the present work was completed (Neuberg & Mandl, 1946).

The diacetone compound. Following our experience of the somewhat unsatisfactory osazone derivative, one of us (D.J.B.) has devised a method by which fructose can be isolated in high yield as its 2:3·4:5-di-*iso*propylidene derivative (Bell, 1947).

The procedure adopted for use with the deproteinized blood was as follows (all concentrations and evaporations were done under reduced pressure, below 35°). The ethanol-chloroform filtrate (1 to 5 l.) was evaporated to small bulk, 2 g. of Hyflo Supercel added and evaporation continued, the solid residue being dehydrated by distillation with ethanol-benzene, and finally kept for some hours in a high vacuum over H_2SO_4 and KOH. The dry mass resulting from the above procedure was broken up, if necessary, and shaken with 50 ml. of acetone (dried over $CaCl_2$) to which had been added 2·5 ml. of conc. H_2SO_4. After 6 hr. anhyd. Na_2CO_3 was added with vigorous stirring until the original dark brown colour of the fluid had changed to a very pale yellow. The solids were filtered off, washed with acetone, and the combined filtrates evaporated to small bulk in the presence of a few drops of 40% NaOH solution. (This ensures that acidic condensation by products of acetone and H_2SO_4 do not attack the labile *iso*propylidene groups in the sugar derivatives.) 100 ml. of water were then added to the syrupy residue and the whole distilled until all the mesityl oxide (a by-product) had passed over. The residue in the flask (*c.* 50 ml.) was shaken with a little charcoal to remove insoluble acetone by-products, and filtered into a separating funnel. The filtrate was three times extracted with its own volume of chloroform, the extract dried over anhyd. Na_2SO_4 and evaporated to dryness. The residue consisted of 2:3·4:5-diacetone fructose, a little of its 1:2·4:5-diacetone isomer, and 1:2·5:6-diacetone glucose. The former compound is completely stable to 0·1 N-H_2SO_4 at room temperature, while the two latter substances undergo quantitative partial hydrolysis to the 1:2-monoacetone derivatives. The mixture was therefore dissolved in cold 0·1 N-H_2SO_4 and the solution kept for 6 hr., after which time it was rendered alkaline (pH 9) by addition of 40% NaOH solution, shaken with charcoal if deemed necessary, and filtered into a separating funnel. Threefold extraction of the filtrate with its own volume of chloroform quantitatively extracted the 2:3·4:5-diacetone fructose only. After drying over Na_2SO_4 the extract was evaporated to dryness and the resulting crystalline residue recrystallized from ligroin (b.p. 60–80°).

The results of three experiments are summarized in Table 7.

Table 7. *Yields of diacetone fructose from ethanol-chloroform concentrates*

Blood samples	Vol. of filtrate (ml.)	Fructose content of blood sample (mg.)	Crude yield of diacetone compound (mg.)	Crude yield of diacetone compound (% of theory)	After recrystallization m.p.	After recrystallization $[\alpha]_D$ (H_2O)
8, 9, 10	1200	136	140	71	94–95°	−32·95°
3, 4, 5, 6, 7, 13, 15	2500	280*	353	87	95°	−32·80°
12, 14, 16, 17	4900	470†	480	71	95°	−32·90°

Fructose : non-fructose ratios in cadmium filtrates: *1·3 : 1, †1·2 : 1.

Bearing of these results on the analyses of cadmium filtrates

Relation of results on ethanol-chloroform concentrates to those on cadmium filtrates. The main difference between the two types of deproteinization is in the degree to which non-fermentable reducing substances are removed. The non-fermentable reducing substances in the ethanol-chloroform filtrates are a considerable fraction of the t.r.s. With human blood and adult sheep blood the cadmium method removes effectively all this material, which includes such substances as glutathione, uric acid, etc. The question therefore arises as to the validity of assuming that the same is true of the foetal blood, and that the difference between fructose by Seliwanoff, and t.r.s. by ferricyanide-cerium is to be attributed to glucose.

That this might not be the case was shown by the fructose : glucose ratios obtained by Seliwanoff and glucose oxidase with ethanol-chloroform concentrates of bloods 3, 4, 5, 6, 7 (pooled) and 19. In the first this was rather greater than 2 : 1 (52·0 : 24·3) and in the second 8 : 1 (104·1 : 13·2). The fructose : non-fructose t.r.s. ratios of the cadmium filtrates were respectively 56·0 : 48·8, and 123·6 : 39·2. In each case the recoveries of fructose are good (see Table 1), and the discrepancy could be due either to an incomplete recovery of glucose in the ethanol-chloroform filtrate, or to the presence of non-fermentable reducing substances in the cadmium filtrate. The latter possibility was tested directly on other blood filtrates.

Glucose oxidase treatment and yeast fermentation of cadmium filtrates. It was found in preliminary experiments that both glucose oxidase and yeast would work in the presence of cadmium filtrates to which phosphate buffer had been added.

For these experiments blood was collected into a glass-stoppered flask containing potassium oxalate. 10, 5 and 1 ml. samples were withdrawn, treated with 80, 40 and 8 ml. of acid cadmium sulphate solution, and shortly afterwards with 10, 5 and 1 ml. of NaOH, of a concentration adjusted to give complete precipitation of the cadmium. The 1 ml. sample was heated in a boiling water bath for 2 min., cooled and filtered. The other two were centrifuged and the clear supernatant liquid decanted through filter papers into stoppered flasks. 5 ml. of 0·5 M-phosphate buffer (pH 6) were diluted to 50 ml. with the unheated cadmium filtrate, and used for the experiments in Table 8. No precipitation was noticed in this solution, which remained perfectly clear on standing for several hours. A second series of experiments was done to compare the heated and unheated cadmium filtrates, particularly with respect to non-fermentable reducing substances. The results of these are summarized in Table 9.

The discrepancy between the t.r.s. estimations on the filtrate with and without added glucose (6, 4), indicates that the glucose oxidase had not worked to completion in the former, and the figures for glucose in the filtrate must therefore be regarded as a minimum. Unfortunately no more foetal blood was available for a repetition of the experiment, this being the last foetus of the 1946–7 season. This was not thought to justify delaying publication for another year, although it detracts from the value of the results here recorded.

Table 8. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a cadmium filtrate of blood sample* 20

No. of exp.	Blood filtrate + buffer (ml.)	Water filtrate + buffer (ml.)	Yeast suspension (ml.)	Glucose oxidase + catalase (ml.)	Added sugar	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
1	—	—	—	—	—	55·2	82·4
2	20·0	—	10·0	—	—	1·1	14·8
3	—	20·0	10·0	—	2 mg. fructose	1·1	8·0
4	20·0	—	—	0·2	—	54·5	63·6
5	—	20·0	—	0·2	—	—	3·4
6	5·0	—	—	0·05	1 mg. glucose*	57·2	76·0

* The added glucose would represent an additional 200 mg./100 ml. of the original blood, i.e. about 10 times that in 4.

Table 9. *Effect of heating cadmium-precipitation mixture on the amount of non-fermentable t.r.s.*

No. of exp.	Cadmium filtrate of blood (ml.)	Cadmium filtrate of water (ml.)	Yeast suspension (ml.)	0·1 M-Phosphate buffer, pH 6 (ml.)	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
7	5·5*	—	3·0	1·0	2·5	26·4
8	—	6·0	3·0	1·0	—	14·0
9	6·0	—	3·0	1·0	1·4	23·8

* Heated 2 min. at 100°; plus 0·5 ml. water.

From these results the following calculations can be made:

The relation between non-fructose fermentable t.r.s., and glucose by glucose oxidase.

From (1), (2), (3) we have $82{\cdot}4-(14{\cdot}8-8{\cdot}0)=$ 75·6 mg./100 ml. fermentable t.r.s.

From (1), (4), (5) we have $82{\cdot}4-(63{\cdot}6-3{\cdot}4)=$ 22·2 mg./100 ml. glucose.

From (1), (2), (3) we have $55{\cdot}2-(1{\cdot}1-1{\cdot}1)=$ 55·2 mg./100 ml. fermentable fructose.

I.e. non-fructose fermentable t.r.s. $75{\cdot}6-55{\cdot}2=$ 20·4 mg./100 ml. Glucose by glucose oxidase = 22·2 mg./100 ml.

The difference between these two figures (1·8 mg./100 ml. of the original blood) is within the limits of experimental error.

The non-fermentable t.r.s. in the cadmium filtrate. This is equivalent (as hexose) to 6·8 mg./100 ml. (2, 3), 12·4 mg./100 ml. (7, 8), and 9·8 mg./100 ml. (9, 8). These values, which are similar to those obtained in an earlier experiment, could well be disregarded in the estimation of t.r.s. in the cadmium filtrate, but they are of the same order as the glucose content, and 'inflate' this if it is assumed to be equivalent to (t.r.s. – fructose). This result confirms the earlier supposition based on experiments with ethanol-chloroform concentrates, and it would seem that when a critical investigation of the glucose and fructose levels is being conducted, the use of glucose oxidase or some other method specific at least for aldoses, is desirable.

DISCUSSION

The experiments here described establish beyond doubt that all, or practically all, the substance in the blood of the foetal sheep giving a positive Seliwanoff reaction is D-fructose. They further establish an equally important fact, that this fructose is present mainly in the free state. This could not be inferred from the Seliwanoff reaction, nor from the formation of the diacetone derivative, nor even from its removal by yeast, but is clear from the formation of a methylphenylosazone, and from the changes in optical rotation accompanying yeast treatment. We believe that this is the first unequivocal demonstration of the presence of free fructose in blood.

In addition, the experiments provide strong evidence that apart from free fructose the blood also contains free glucose, but in much smaller concentrations in most of the samples examined. Within the limits of the methods employed there was no evidence for the presence of any other fermentable reducing sugar. This is confirmed by the findings of Partridge (1948) with the paper partition chromatogram; he detected glucose and fructose, but no appreciable quantities of any other reducing sugar.

It will be noticed that neither the glucose nor fructose, nor the two combined, are apparently maintained at a constant level, as one is accustomed to find the blood sugar of some animals. This may be due in part to the fact that the samples were taken from anaesthetized animals (cf. Hitchcock, 1948).

The proof that fructose is a constituent of foetal blood helps to explain the finding of Passmore & Schlossmann (1938) that the blood sugar level of the foetal sheep was higher than that of its mother (also in the case of the goat, and in the cow and pig; Aron, 1924). It seems probable from our results, and those of Cole & Hitchcock (1946) that the blood *glucose* (which should be if anything less than the non-fructose t.r.s.) of the foetus is lower than that of its mother, which would be more in keeping with results in other animals (cf. Needham, 1931), although of course this is no reason for assuming that fructose does not occur in the foetal blood of these species also. Orr (1924) states that Paton *et al.* (1907) demonstrated the presence of fructose in the blood of both foetal sheep and cow, but we have not been able to find any direct reference to either animal in the original paper. Orr himself showed that there was a fructose-like substance in human foetal blood, and in that of a kid just after birth, but from the feeble negative rotation of his blood concentrates he concluded that more glucose than fructose was present.

By the use of the methods we describe it should be possible to identify and estimate fructose in a variety of foetal bloods, and thus discover whether its occurrence is general. It might be expected that this is so from the general occurrence of Seliwanoff-positive substances, almost certainly fructose, in the amniotic and allantoic fluids of a wide range of species. At birth it would seem that the fructose is quickly lost, a part, or all being eliminated in the urine (cf. Langstein & Neuberg, 1907; Cole & Hitchcock, 1946).

It is too early to attempt an explanation for the presence of fructose in the foetus, or, for that matter, for the equally obscure and abrupt transference of the young animal at birth to a diet containing lactose, although the suggestion of Mann (1946), that fructose provides a source of energy for spermatozoa which is not shared by the somatic tissues, may have some bearing on this problem also.

SUMMARY

1. The Seliwanoff-positive substances in the blood of the foetal sheep have been accounted for by the presence of free D-fructose: they are fermentable, have a negative optical rotation closely corresponding to that of D-fructose, yield a methylphenylosazone under conditions in which glucose forms none, and give the expected high yield (70%)

of 2:3-4:5-di-*iso*propylidene-D-fructopyranose on treatment with acid acetone under suitable conditions.

2. The reducing substances other than fructose are partly fermented by yeast, and this fraction has been accounted for by D-glucose, which was estimated by the disappearance of reducing substances on treatment with the specific enzyme, glucose oxidase.

3. A small part of the reducing substances in solutions deproteinized by the use of acid cadmium sulphate and sodium hydroxide, and a greater part of those in solutions deproteinized by chloroform and ethanol, are not fermented by baker's yeast; no evidence was obtained for the presence of any reducing sugar other than D-fructose and D-glucose.

4. The implications of these findings are discussed.

We wish to place on record our gratitude to the late Sir Joseph Barcroft, F.R.S., for the opportunity to investigate this problem, and for his encouragement, given so generously and in so many ways. We are indebted to Dr T. Mann and Mr S. W. Cole, for their advice and help in the course of the work; to Miss V. Moyle, Mr M. W. S. Hitchcock, and Dr S. M. Partridge for analyses which they performed for us; and to the members of the Agricultural Research Council Unit of Animal Physiology for their friendly co-operation.

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Physicochemical Observations on some Glycogens

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The molecular weight of unsubstituted glycogen has, up to the present, been studied in but a few instances. Oakley & Young (1936) have reported, for samples from rabbit liver and muscle, values of $1{\cdot}2$–$2{\cdot}3 \times 10^6$ for the former, and $0{\cdot}7$–$1{\cdot}8 \times 10^6$ for the latter material. These results were obtained by use of a delicate method of osmotic-pressure measurement on solutions in 0·1N-calcium chloride. These authors claim that both alkaline extraction of the tissue (the so-called Pflüger method) and water extraction followed by precipitation by acetic acid (Bell & Young, 1934) yield products of the same general molecular size. Oakley & Young (1936) further noted that glycogen solutions when salt-free manifested markedly greater osmotic pressures than in presence of Ca^{++} and Cl^-. Bridgeman (1942) determined the sedimentation and diffusion constants of rabbit-liver glycogen and derived from these an average molecular weight of about 4×10^6. Glycogen from tubercle bacilli has been shown by Chargaff & Moore (1944), using similar methods, to have a molecular weight of 12–13×10^6. These are the only reported molecular weight determinations using the ultracentrifuge.

Our object in the present work was to compare certain physical properties of a number of glycogen preparations.

EXPERIMENTAL AND RESULTS

Preparation of material. Preparations were made from the appropriate tissue either by water extraction or by the Pflüger technique. In each instance final purification was effected by fourfold precipitation by acetic acid (Bell & Young, 1934). Relevant data are summarized in Table 1.

Viscosity measurements. These were made in an Ostwald-type viscometer having a flow time for water of about 3 min. Series of solutions of each sample of glycogen, in concentrations ranging from 0·2 to 2·0%, were examined. Except where indicated in Table 2, we observed no marked dependence of the specific viscosity upon concentration.

Diffusion rates. These were observed on 1% solutions (in M-NaCl) of the glycogens in a cell of the type described by Lamm & Polson (1936). Diffusion constants were calculated by the inflexion-point method and distribution curves over the boundary were obtained by the Philpot (1938) optical system. The diffusion constants (D_{20}) recorded in Table 2 are corrected to diffusion in water at 20°.

Sedimentation rates. The sedimentation velocities were observed in the Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938). Fig. 1 shows specimen diagrams. The thicknesses of the sedimenting boundaries were in all instances much greater than could be accounted for by diffusion, showing that the samples were markedly polydisperse. The glycogen of rabbit liver prepared by the Pflüger method (sample IV) was more polydisperse than that made by water extraction (sample V). The mean sedimentation constants, corrected to the values for water at 20° (S_{20} corr.), are given in Table 2.

Partial specific volume. V was calculated both from the densities of aqueous solutions and from the density of dry glycogen samples using diethyl β-ketoglutarate. (This liquid was chosen for the reasons that it is not appreciably volatile, has relatively low viscosity and rapidly wets glycogen without dissolving it.) There appear to be no

Table 1. *Glycogen specimens examined*

Serial number	Source	Method of isolation (P, by Pflüger technique; W, by water)	No. of residues in average unit chain	References to previous work on specimen used
I	Horse muscle	P	11–12	Bell (1937)
			11–13	Bell (1944)
II	Rabbit muscle	P	12	Bell (1948)
III	Human muscle	P	11	Halsall, Hirst & Jones (1947)
IV	Rabbit liver	P	12	Bell (1935)
V	Rabbit liver	W	12	Bell (1935)
VI	*Ascaris lumbricoides*	P	12–13	Baldwin & King (1942)
			13–14	Bell (1944)

Table 2. *Molecular weights of glycogen specimens*

Specimen	Solvent	$\frac{\eta/\eta_0 - 1}{C}$	$D_{20} \times 10^7$	S_{20} (corr.) $\times 10^{13}$	Mol. wt.* from S_{20} and D_{20}	Mol. wt. from osmotic pressure
I	Water	0·074	—	—	—	—
	M-NaCl	—	1·50	63	$2{\cdot}9 \times 10^6$	—
II	Water	0·074	—	—	—	—
	M-NaCl	0·055	1·55	58	$2{\cdot}6 \times 10^6$	—
III	Water	0·060	—	—	—	—
	M-NaCl	—	1·65	57	$2{\cdot}4 \times 10^6$	—
IV	Water	0·120–0·065	—	—	—	$0{\cdot}5 \times 10^6$
	M-NaCl	—	1·27	81	$4{\cdot}4 \times 10^6$	$2{\cdot}0 \times 10^6$
V	Water	0·075–0·063	—	—	—	—
	M-NaCl	—	1·21	75	$4{\cdot}3 \times 10^6$	—
VI	Water	0·135	6·25	—	—	$0{\cdot}1 \times 10^6$
	M-NaCl	—	1·80	18	$0{\cdot}70 \times 10^6$	$0{\cdot}5 \times 10^6$

* Calculated from the equation mol. wt. $= \frac{RTS}{D(1 - V\rho)}$, where R = gas constant; T = absolute temperature; S = sedimentation constant; D = diffusion constant; V = partial specific volume and ρ = density of solvent.

significant differences between the densities of glycogen samples from different sources, and the calculated partial specific volume was equal to 0·64–0·66.

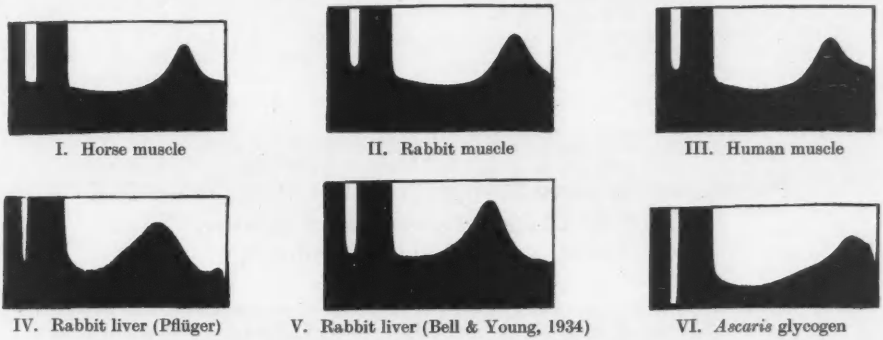

Fig. 1. Sedimentation diagrams. The following are the values of the speed of rotation (rev./sec.) and the time after reaching full speed at which the diagrams were obtained (roman numbers refer to the glycogen specimens: see Table 1). I, II, III: 450; 25 min. IV: 400; 30 min. V: 315; 60 min. VI: 625; 20 min. Concentration of glycogen: in each case 1 g./100 ml. of solvent.

Osmotic pressure. This was measured by the method of Adair (1925). The concentration of glycogen after equilibrium was determined polarimetrically. A series of experiments over a range of concentrations of from 1 to 7 % of solute showed that the apparent molecular weight calculated from osmotic pressure is markedly dependent on concentration. It was difficult to apply the extrapolation to zero concentration recommended by Adair & Robinson (1930) as the pressure exerted by dilute solutions was very small and the results obtained yielded, therefore, only rough approximations.

DISCUSSION

From the observations recorded in Table 2, it is clear that glycogens rank among the largest known metabolites, and that significant differences of molecular size occur according to the source from which the glycogen is obtained. Although all the samples were polydisperse and although the degree of polydispersity evidently depends on the method of isolation, their general uniformity suggests that these values may be valid for glycogen as it occurs in the cell.

The lower values of molecular weight obtained by osmotic measurement are to be expected in view of the polydispersity of the material, since osmotic pressure depends on the 'number average' while sedimentation and diffusion depend on the 'mass average' of molecular species.

The lowering of the osmotic pressure by salts, already noticed by Oakley & Young (1936), is difficult to account for. Disaggregation is unlikely in view of the lowering of viscosity produced by salt. A Donnan lowering is unlikely in view of the absence of ionizable groups in glycogen, though compensation of a charge-effect would account also for the lowering of viscosity. The effect of salt on viscosity might be accounted for by a decreased asymmetry of the molecule; this might be accompanied by a reduction of the number of its possible configurations which would account, on statistical grounds, for the reduction of osmotic pressure.

SUMMARY

1. The average molecular weights of samples of muscle glycogen have been shown, from sedimentation and diffusion data, to be of the order of $2{\cdot}4–2{\cdot}9 \times 10^6$.

2. Samples of liver glycogen have molecular weights of $4{\cdot}4–4{\cdot}3 \times 10^6$. Alkaline extraction of the glycogen from its parent tissue does not effect very serious degradation.

3. Glycogen from the whole tissues of *Ascaris lumbricoides* has a much smaller molecular weight, namely $0{\cdot}70 \times 10^6$.

4. Osmotic pressure measurements have been shown to lead to estimations of molecular weight considerably smaller than those afforded by sedimentation-diffusion methods.

5. In all instances the glycogens were found to be polydisperse with respect to molecular size.

6. The observations of Oakley & Young on the effect of salts on the apparent molecular weight by osmotic pressure have been confirmed.

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Partial Amino-acid Compositions of some Plant-leaf Protein Preparations: the Arginine, Histidine and Lysine Contents

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In an earlier publication (Lugg & Weller, 1944) the amide, tyrosine, tryptophan, cyst(e)ine (i.e. cystine plus cysteine calculated as cystine) and methionine contents of some extracted plant-leaf protein preparations were reported. These preparations, one each from leaves of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., were judged, both from the mode of preparation and from analytical evidence, to be reasonably representative of the whole proteins in the leaf materials. The arginine, histidine and lysine contents of these same preparations have now been estimated and it is one purpose of this article to record the values obtained. The question of the existence of hydroxylysine in these preparations has also been considered.

EXPERIMENTAL

Methods. The arginine and lysine contents of the preparations were estimated by methods based essentially upon those of Tristram (1939). Histidine contents were at first estimated by Tristram's (1939) method, but for reasons to be discussed, the resulting values were considered unsatisfactory and recourse was had to an adaptation of the method of Vickery & Winternitz (1944). A modification of the Kapeller-Adler (1934) colorimetric method of estimating histidine, recommended by Block (1937), was unsatisfactory in our hands.

Hydrolysis. In Tristram's experience the hydrolysis of plant-leaf protein preparations for the purpose of estimating the contents of these three amino-acids could be performed satisfactorily by heating the protein with about ten times its weight of 4 M-H_2SO_4 for 18–22 hr. at the boiling point of the mixture (approx. 110°). Whilst it is unlikely that these conditions would secure the maximum 'net production of amide NH_3 plus amino-acid "amino" groups' (see Lugg, 1946), they may achieve almost complete liberation of these particular amino-acids from such proteins. In our work, about 2·7 g. of the air-dried preparation (about 2·5 g. dry wt.) were heated for *c.* 1 hr. with 25 ml. of 4 M-H_2SO_4 at 100° and then at the boiling point of the mixture for 21–22 hr., which is the upper region of Tristram's (1939) range.

Estimation of arginine, histidine and lysine. The excessive variability of replicate histidine values estimated by Tristram's (1939) method and the extreme uncertainty of values obtained by the Conrad & Berg (1937) modification of Kapeller-Adler's (1934) method (tints of standard and test colour solutions frequently differed markedly) made it desirable to find a more satisfactory procedure. The Vickery & Winternitz (1944) method, in which the histidine is estimated gravimetrically as the di-3:4-dichlorobenzene sulphonate (histidine-DBS), was adapted for use in a general procedure for the estimation of arginine and histidine.

In this procedure insufficient Ag_2O and H_2SO_4 for precipitation of Ag_2SO_4 to occur were used, and larger quantities of $AgNO_3$ than are needed in Tristram's (1939) procedure, had to be employed. This and subsequent steps were taken to prevent the introduction of variable amounts of H_2SO_4 and/or HCl, in the presence of which we found the solubility of histidine-DBS to be appreciably increased. Urea was added in small quantities to decompose any nitrous acid which may have been present (from decomposition of nitrate by reducing substances in acid solutions). Tristram's procedure was then followed for the separation of the arginine- and histidine-silver compounds and for the isolation of the arginine and lysine as monoflavianate and monopicrate, respectively.

The histidine was liberated from the silver compound by treatment with H_2S (Vickery & Winternitz, 1944, used HCl) and was then precipitated as histidine-DBS in the

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manner described by these authors, but from a volume of 10 ml. and with 1·5 g. of the acid. The salt was recrystallized repeatedly from aqueous solutions of the acid until the recrystallization loss was the same as that found for the pure salt. To the final weight were added the recrystallization losses of the pure salt for every recrystallization. Purity was confirmed by melting-point determination. A correction for overall losses of +2·1 mg. of histidine N was made (see later references).

The arginine monoflavianate was recrystallized repeatedly from water and the final weight of pure salt was corrected for the recrystallization losses. A correction for overall losses of +0·4 mg. N was made (see later references) in addition to the correction for solubility of the arginine-silver compound. This correction, which is much smaller than Tristram's, was applied also to results obtained by his unmodified method. Incidentally, Chibnall, Rees & Williams (1943) believed the earlier correction to be too high. Weights of crude salt obtained by Tristram's unmodified method were corrected for impurity by multiplying by a factor, 0·965, derived by recrystallizing samples obtained in several analyses and pooled samples from the other analyses.

The lysine monopicrate was tested for purity by fractional recrystallization of mixed samples and chromatographing the fractions; a general correction was then applied by multiplying the weights of crude material by 0·975. Tristram's (1939) correction for overall losses (+1·6 mg. N) was made.

Arginine, histidine and lysine were thus estimated by Tristram's (1939) methods (method 1), but histidine (and, in some instances, arginine) were estimated also by the procedures outlined above (method 2).

Losses of arginine, histidine and lysine in the analytical procedures. In the examination of losses, mixtures of the following quantities of amino-acids were used: approx. 0·12 g. L-arginine, 0·04 g. L-histidine, 0·12 g. L-lysine, 1·0 g. glycine, 0·5 g. DL-alanine and 0·5 g. DL-aspartic acid. These mixtures, dissolved in 25 ml. of 4M-H_2SO_4 solution, were (*a*) submitted to direct analysis, (*b*) 'hydrolyzed' at the boiling point for 21–22 hr. and then analyzed, (*c*) treated with 0·5 g. of L-arabinose before 'hydrolysis' and analysis.

Table 1. *Percentage losses of arginine* (0·12 *g.*) *and histidine* (0·04 *g.*), *when submitted with lysine* (0·12 *g.*), *glycine* (1·0 *g.*), *alanine* (0·5 *g.*) *and aspartic acid* (0·5 *g.*) *to the hydrolysis procedure in the absence and in the presence of arabinose*

Arabinose present (g.)	Losses (%)	
	Arginine	Histidine
0	0	0
0·50	16	4

From replicate (*a*) experiments were found the mean corrections for overall losses (previously given) for use with method 2. From replicate (*b*) and (*c*) experiments were derived the data shown in Table 1. In analytical procedures (such as these) which involve the separation of amino-acid salts of moderate solubility, losses might be expected to arise from any racemization suffered by the amino-acids. The results shown in Table 1, and those of Tristram (1939) for lysine, indicate that to whatever extent racemization might have occurred, recoveries of the amino-acids could not have been affected by it. Losses in absence of carbohydrate, estimated by other methods also, appear to be zero for all three amino-acids concerned (see Chibnall, 1946).

Tests with regard to the possible presence of hydroxylysine in the protein preparations

Van Slyke, Hiller, Dillon & MacFadyen (1938) have described the isolation of lysine and hydroxylysine picrates from gelatin hydrolysates by a procedure which differs very little from that used in this work (above) for the isolation of lysine monopicrate, the main points of difference being that they repeatedly recrystallized the phosphotungstates to remove other amino-acids, and fractionally precipitated the picrates. They state that hydroxylysine picrate is rather more soluble than the lysine salt in water. From their work it may be presumed that, if the leaf-protein preparations were to contain appreciable quantities of hydroxylysine, its picrate would contaminate the crude lysine salt isolated in this work.

Samples of crude lysine monopicrate obtained in analyses of the four protein preparations were mixed in approximately equal amounts and submitted to fractional recrystallization from aqueous solution. A disgusting odour, suggestive of mustard and CCl_4, was evolved when the mixture was dissolved in hot water. By repeated fractional recrystallization five fractions were obtained. The head fraction accounted for 85·1% of the weight of the mixture, successive fractions for 1·6, 3·4 and 3·3, and the tail fraction 6·1%; 0·5% was not recovered, and may have been lost in the effluvium referred to above.

The five fractions were submitted to so-called 'one-dimension' partition-chromatographic analysis (Consden, Gordon & Martin, 1944). The stationary phase was phenol-saturated water containing 0·3% (w/v) of NH_3, and the mobile phase was water-saturated phenol. Preliminary liberation and separation of the amino-acids from the picric acid was found to be unnecessary, a picrate (NH_4 salt?) band travelling independently of the amino-acids. From the intensities of the band colorations it was estimated roughly that the amino-acids in the original mixture of crude picrates probably contained 98% of lysine. Of the remainder, only a small fraction, if any, could have been hydroxylysine.

RESULTS

The results of the analytical work on the protein preparations are given in Tables 2 and 3. In Table 2 are shown the percentages of the leaf-protein nitrogen found in the form of arginine, histidine and lysine in the hydrolysates with assessments of the random errors of estimation. The table is self-explanatory.

Evaluation of the analyses

It seems likely that method 2 would have given, if applied generally, arginine values similar to those obtained by method 1, but with considerably smaller variability of replicate estimations (to judge from the work with the test mixtures). The relatively large variability of replicate arginine estimations by method 1 is attributable, at least in part, to the fact that, although the degree of purity of the isolated crude arginine monoflavianate must have varied,

Table 2. *Percentages* of protein N found in the hydrolysates in the forms of arginine, histidine and lysine (corrected only for systematic errors of analysis of hydrolysates), and other data*

Protein prep.†		N in prep.‡ (%)	Ash in prep.‡ (%)	Arginine N (%)		Histidine N (%)		Lysine N (%)
				Method 1	Method 2	Method 1	Method 2	
1E	*Phalaris tuberosa*	14·90	1·8	12·5 (5)	—	2·10 (4)	3·58 (2)	6·60 (3)
2E	*Hordeum murinum*	14·10	2·8	12·6 (4)	—	2·00 (4)	3·52 (3)	6·33 (4)
3E	*Medicago sativa*	14·15	2·3	12·2 (2)	12·4 (2)	2·16 (2)	3·65 (3)	6·36 (2)
4E	*Medicago denticulata*	14·55	1·4	11·6 (3)	—	2·00 (3)	3·86 (2)	6·51 (3)
'Within preparations' estimate of standard deviation				[0·42]	[0·14]	[0·229]	[0·034]	[0·303]

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

† The serial cyphers are those assigned by Lugg & Weller (1944). ‡ Data from Lugg & Weller (1944).

Table 3. *Percentages* of the protein N occurring as arginine, histidine and lysine, the values having been corrected for hydrolysis losses believed to result from the presence of impurity*

Protein prep.		N in prep. (%)	Arginine N (%)	Histidine N (%)	Lysine N (%)
1E	*Phalaris tuberosa*	14·90	13·7 (5)	3·66 (2)	6·60 (3)
2E	*Hordeum murinum*	14·10	14·3 (4)	3·63 (3)	6·33 (4)
3E	*Medicago sativa*	14·15	14·0 (4)	3·76 (3)	6·35 (2)
4E	*Medicago denticulata*	14·55	13·0 (3)	3·97 (2)	6·51 (3)

Analysis of variance

	Arginine			Histidine			Lysine		
Source of variation	D.F.	S.S.	Mean square	D.F.	S.S.	Mean square	D.F.	S.S.	Mean square
Between preparations	3	3·21	1·07	3	0·1521	0·0507	3	0·1526	0·0509
Within preparations	12	2·24	0·187	6	0·0070	0·0012	8	0·7325	0·0916
Variance ratio			5·7			42			0·56

As judged by the *F* variance ratio, there is a significant difference between the mean squares for arginine, a highly significant difference between those for histidine, and no significant difference between those for lysine (see discussion).

* In parentheses beside the values are indicated the numbers of estimations from which the values (the means) were derived.

a uniform correction factor was applied to the weights of crude salt in most of the analyses. It is clear that method 2 has yielded consistently larger histidine values than method 1.

Even if the correction for overall losses (+2·1 mg. N) were not applied, the values for histidine obtained by method 2 would still be much greater than the corrected values by method 1. Obviously, the histidine values obtained by method 1 cannot be accepted. The only explanation which seems to be in keeping with the careful examination, which Tristram (1939) made, is that, whereas at the stage of precipitation of the histidine diflavianate his test solutions probably contained very little amino-acid other than histidine, those derived from the protein hydrolysates probably contained rather more, and that the solubility of the histidine salt must be vastly increased in the presence of the other amino-acids. Our test mixtures contained large quantities of other amino-acids, but in any case Vickery & Winternitz (1944) have adduced reasons for believing that the solubility of histidine-DBS is probably only slightly affected by the presence of other amino-acids. Attention must be drawn to the unusually small variability of replicate histidine estimations made by method 2.

There is some uncertainty about the accuracy of the lysine values. Chibnall, Rees & Williams (1943) obtained good agreement between lysine values for β-lactoglobulin estimated by Tristram's (1939) method and by their own comprehensive method in which, likewise, lysine monopicrate is weighed. However, a comparison of these values with those obtained by isotope dilution, microbiological assay, specific decarboxylase, and electrodialysis (with N difference) methods, shows that the former are almost certainly too low (see Macpherson, 1946). Chibnall (1946) has given comparisons for some other proteins, the agreement being excellent in some instances and poor in others. In Tristram's (1939) method the initial precipitation of the lysine monopicrate occurs under such conditions (high concentration of ethanol) that the solubility of the salt is probably very low, whether other picrates are present or not. Our assessment of the purity of the crude lysine monopicrate was not based upon recrystallization losses. Consequently, if enhanced recrystallization losses constitute the major source of systematic error in such estimations, our values may be fairly reliable.

In compiling Table 3 we have returned to the individual values from which the mean values shown in Table 2 were derived. Of the histidine values only those obtained by method 2 have been used. All the arginine values have been used. Those for 3E obtained by both methods have been grouped, as they differed but little among themselves, and the inclusion of the two values obtained by method 2 could not appreciably affect a general statistical analysis based upon the supposition that all values were obtained in the same way. The individual values have then been corrected

on the following basis. The assumption has been made that during hydrolysis the protein preparations yielded all of these three amino-acids contained in them, that the destruction in the absence of carbohydrate or polyuronide impurities would not have been any greater than in the test 'hydrolysis' experiments (i.e. 0%), because the bulk of the amino-acids is probably liberated within the first several hours of hydrolysis (see Lugg, 1946), that the losses due to the presence of such impurity are proportional to the amount of it, that the amount can be calculated with sufficient precision from the known N contents of the preparations (corrected for ash) and the N contents (believed to be about 16·75%; see Lugg, 1939) of the hypothetical pure proteins, and that 1·00 g. of arabinose corresponds in quantity and effects with 0·88 g. of (pentosan) impurity (Lugg, 1939).

The impurities present in 2·5 g. quantities of the four preparations were thus calculated to have been equivalent in effects to the following quantities of arabinose: 0·27 g. (1E); 0·38 g. (2E); 0·39 g. (3E); 0·34 g. (4E). The corresponding corrections for the arginine and histidine values were calculated from the data in Table 1. On the basis of Tristram's (1939) work, no corrections were required for the lysine values.

Lugg (1939) was of the opinion that the common impurity in preparations like these may be pectin or more probably mucilage, rather than pentosan, and the corrections calculated on the above basis may require revision if, at some future time, recovery hydrolysis experiments are made with these other possible contaminants. The means of the corrected values are shown in Table 3. The corrected (individual) values were used in the analysis of variance given at the foot of the table.

DISCUSSION

Although an element of uncertainty must yet remain with respect to destruction of amino-acids during their hydrolytic liberation from proteins, recent extensive analyses of several 'pure' proteins (reviewed by Chibnall, 1946) suggest that the destruction, in most instances, is probably small. The results given in Table 3, in which corrections have been made for assessable losses, are to be viewed in the light of these remarks.

The arginine and lysine contents of the four protein preparations are of similar magnitudes to those found by Tristram (1939) for plant-leaf protein preparations which (for the most part) were probably less fully representative of the total protein in the leaves, but there is reason to believe that Tristram's (1939) arginine values may have been somewhat overestimated (see earlier remarks concerning methods and overall losses). The histidine contents of the four preparations are very considerably greater than those found by Tristram (1939) for the other preparations. It is to be noted, however, that when the method used by Tristram (1939) was applied to the four preparations, values similar to those he reported for the other preparations were obtained. For reasons already discussed, we have discarded the histidine values obtained by Tristram's (1939) method. We are of the opinion that the histidine values reported by Tristram (1939, 1940), and by Lugg (1943) who used the same method in an analysis of protein from the fronds of *Pteridium aquilinum*, were probably too low. Indeed, it seems likely, in the light of the discussion in the previous section, that almost all protein histidine contents obtained by the use of methods involving precipitation as diflavianate, whether they be large- or small-scale procedures, will require re-examination. This is implied also by the results of recent electrodialysis studies (Macpherson, 1946).

Attention has been drawn (Lugg & Weller, 1944) to the fact that the cyst(e)ine and methionine contents of the legume leaf proteins (3E, 4E) are appreciably lower than those of the grass leaf proteins (1E, 2E), and that the same tendency had been observed among protein preparations, many of which were probably far less fully representative of the total protein in the tissues. An interesting point arising from the present study is the astonishing uniformity in the arginine, histidine and lysine contents of the four preparations. Statistical examination of the values, with the aid of the analysis of variance given at the bottom of Table 3 and the *t* test, reveals that at the 95% probability level the differences in arginine values are significant only as between 4E and the other preparations, whereas the differences in histidine values, small though they are, are significant in all cases except as between 1E and 2E. However, the differences and their degrees of significance would be modified somewhat by the incidence of errors in the reported N contents of the protein preparations.

SUMMARY

1. The arginine, histidine and lysine contents of protein preparations, 1E, 2E, 3E and 4E, which are believed to be reasonably representative of the total protein in leaves respectively of *Phalaris tuberosa* L., *Hordeum murinum* L., *Medicago sativa* L., and *M. denticulata* Willd., have been estimated. As percentages of the protein N occurring in the forms of these three amino-acids, the values for the four plant species, respectively, may be expressed: arginine, 13·7, 14·3, 14·0, 13·0; histidine, 3·66, 3·63, 3·76, 3·97; lysine, 6·60, 6·33, 6·36, 6·51. The work involved an examination of methods of analyzing hydrolysates, and an examination of the probable effects of impurities in the protein preparations upon the analyses.

2. It is believed, on the evidence afforded by fractional recrystallization of amino-acid picrates and 'one-dimensional' partition-chromatographic analyses of the fractions, that the preparations contained no hydroxylysine.

We wish to thank Prof. M. H. Belz for assistance with the statistical examination of the data.

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Protein in Senescent Leaves of *Trifolium subterraneum*: Partial Amino-acid Composition

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It has been shown (Lugg & Weller, 1941) that in very young leaf tissue the whole protein composition may differ rather markedly from that of the whole protein in older tissue. (The axial organs of germinating seeds were used, but the bulk of the protein was present in the stalks and plumules.) In the following account it will be shown that at the other extreme of age (senescent tissue) the departure in composition of the whole-leaf protein from the pattern which seems to prevail during most of the life of the plant may again be fairly marked in some respects.

EXPERIMENTAL

Preparation of materials. The fresh plant material consisted of the leaves and attached petioles of *Trifolium subterraneum* harvested very late in the season (December, 1940) from senescent plants growing in a relatively cool and damp environment in the Adelaide foothills. 'Yellowing' was somewhat advanced in about a third of the leaf material, and much of the rest showed traces of it.

From the bulk of the fresh material an 'extracted' protein preparation (E) was made by the alkaline buffer lipid-solvent method (Lugg, 1939) in the manner described by Lugg & Weller (1944). 'Residual' (R) and 'whole' (W) protein preparations were also made by methods previously described (Lugg, 1939).

Data concerning the fresh material and the protein preparations made from it are shown in Table 1. The terms 'coagulable N' and 'protein N' have been explained fully in earlier publications (e.g. Lugg, 1939). For the rest, the table is self-explanatory.

Analytical work. Nitrogen estimations were made by the Kjeldahl method with Se catalyst. The amide, tyrosine, tryptophan, cyst(e)ine (i.e. cysteine plus cystine) and methionine contents of the protein preparations, W, E and R, were estimated by methods previously described (Lugg, 1937; 1938*a*, *b*, *c*; Lugg & Weller, 1941). The 'differential oxidation method' was used for the estimation of the cyst(e)ine and methionine contents. Considerable difficulty was encountered with the tryptophan estimations on the W and R preparations because of the unusually intense brown coloration of the alkali-stannite hydrolysates. Corrections for this coloration were so large that the precision of the estimations was seriously impaired.

The arginine, histidine and lysine contents of preparation E were estimated by methods recently described (Lugg & Weller, 1948) and based upon the procedures of Tristram (1939) and of Vickery & Winternitz (1944). These methods are considered to be unsatisfactory for use with preparations

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Table 1. *Extraction of protein from senescent leaves of* Trifolium subterraneum

Fresh wt. taken (kg.)	Dry wt. (%)	N in dry leaf (%)			Extracted N (%)			Protein preparation	N in prep. (%)	Ash in prep. (%)
		Total	Coagulable	Protein	Total	Coagulable	Protein			
12·3	20	2·45	2·05	1·99	—	—	—	W	3·16	2·4
—	—	—	—	—	33	28	26	E	13·15	2·6
—	—	—	—	—	—	—	—	R	2·10	3·5

like W and R. Of the protein N, there were estimated to be present in the hydrolysates, 11·2% by method 1 and 11·0% by method 2 (11·1% mean) as arginine, 4·07% by method 2 as histidine and 5·72% as lysine. In deriving this lysine value, weights of crude lysine monopicrate were corrected by multiplying them by the factor 0·975 determined previously for use in analyses of other leaf-protein preparations (Lugg & Weller, 1948). The arginine and histidine values required correction for losses attributable to the presence of interfering impurity in the protein preparation. The interfering impurity in 2·5 g. of preparation E was calculated from the N and ash contents of the preparation and assumed N content of 16·75% for the hypothetical pure protein (see Lugg & Weller, 1948) to correspond in quantity and effects with 0·55 g. of arabinose, and the above arginine and histidine values were accordingly multiplied by the appropriate factors (see Lugg & Weller, 1948).

RESULTS

The results are shown in Table 2, the partial compositions of the preparations being expressed as percentages of the protein N occurring in amide and amide, tyrosine, tryptophan, cyst(e)ine, methionine and cyst(e)ine plus methionine contents of the preparations, and of the rationale of the method of obtaining preparation E, it is believed that this preparation was reasonably representative of the whole protein in the leaf material.

Viewed broadly, the leaf protein composition appears to be similar to those found for other spermatophytes. Comparison of the contents of Table 2 may be made with those of tables in recent publications (Lugg & Weller, 1948; Lugg, 1946). A significant feature of these other tables is that the cyst(e)ine and methionine contents of the whole proteins in leaf tissue from reasonably mature plants are, in general, somewhat lower for the Leguminosae than they are for any of the other plant families investigated. For 'whole', 'extracted' and 'residual' protein preparations from *Trifolium subterraneum* specifically, Lugg (1943) found the cyst(e)ine-N values to be 1·15, 1·13 and 1·12%, respectively, and the methionine-N values to be 1·32, 1·38 and 1·29%, respectively, the cyst(e)ine-N plus methionine-N values being 2·47, 2·51 and 2·41%, respectively. The cyst(e)ine-N plus methionine-N values for preparations W, E and R of the present study are much the same as these, but it will be noticed that the cyst(e)ine contents are distinctly higher whilst the methionine contents are distinctly lower than those reported for the non-senescent material. Indeed, the cyst(e)ine contents are above the range of values, and (except for the seedling material examined by Lugg & Weller, 1941) the methionine contents below the range of values which have been estimated by this method for all other legume leaf materials. Again, the tyrosine values for preparations W, E and R, which are somewhat higher than those reported for the non-senescent material (Lugg, 1943), are among the highest yet encountered in leaf-protein preparations. The histidine content of preparation E is slightly higher than those of two legume- and two grass-leaf preparations recently examined (Lugg & Weller, 1948).

Table 2. *Partial compositions of protein preparations from* Trifolium subterraneum

		Percentages of protein N								
Preparation	N (%)	Amide N	Tyrosine N	Tryptophan N	Cyst(e)ine N	Methionine N	Cyst(e)ine + methionine N	Arginine N	Histidine N	Lysine N
W	3·16	5·48	2·80	1·85	1·42	1·05	2·47	—	—	—
E	13·15	5·39	2·81	1·80	1·43	1·13	2·56	13·5	4·26	5·72
R	2·10	5·71	2·90	1·65	1·47	1·02	2·49	—	—	—

DISCUSSION

In view of the rather low protein content of the leaf material the yield of 'extracted' protein was not, for the procedure employed, unduly poor, nor was the purity of the preparation (judged from its N content) unexpectedly low. Presumably, therefore, no large fraction of the leaf protein was in a denatured condition. From a consideration of the amino-acids concerned. Except for the tryptophan contents the random errors with the W and R preparations are believed not to have been more than 1·5 times as great as those with preparation E, and these in turn are believed not to have exceeded the following percentages (in parentheses) of the tabulated values: N (0·5, affecting all the other errors), amide (1·5), tyrosine (2), tryptophan (3), cyst(e)ine (4), methionine (5), cyst(e)ine plus methionine (2), arginine (4), histidine (2) and lysine (8). Estimates of the likely limits of random errors in the arginine, histidine and lysine values have been based partly upon the deviations encountered in the present work and partly upon those encountered in earlier work with these methods (Lugg & Weller, 1948). Those for the other values have been derived from much experience with the methods concerned.

From past studies of plant-leaf protein preparations we have come to associate small, and usually rather uncertain, composition changes with different samples of leaf material harvested at different times during most of the life history of the plant. We do not know whether the protein in senescent tissue of

T. subterraneum would always correspond closely in composition with the amide and amino-acid values we have obtained in this one instance. In so far as it may be safe to generalize from the data at present available, the onset of senescence appears to have been accompanied by an increase in the cyst(e)ine content (and possibly in the tyrosine content) and a decrease in the methionine content of the whole protein in the leaf material. We do not know whether the compositions of individual proteins in the leaves have undergone change. It is possible that the only change has been in the proportions of the individual proteins. In this connexion it is pertinent to point out that change in haemoglobin composition with transition from foetal to adult mammalian life (as shown by Vickery (1944) for the histidine contents of foetal and adult bovine haemoglobins) may be due to a change in the ratio of two (or more) haemoglobin components (demonstrated for foetal and adult human material by Andersch, Wilson & Menten, 1944).

Effects of plant age and treatment (such as grazing) upon the cyst(e)ine and methionine contents of leaf proteins were judged by Smith & Wang (1941) to be fairly pronounced, but the validity of their conclusions was questioned by Lugg (1943) on the ground that the analytical methods they used would be highly unreliable with the impure leaf-protein preparations they analyzed. That changes in the cyst(e)ine and methionine contents of the whole protein in the leaves of various plants may occur, particularly at the extremes of plant age, seems very likely, but our earlier work, covering considerable ranges of the intermediate ages of plants, has revealed no definite, progressive changes.

SUMMARY

1. The amide, tyrosine, tryptophan, cyst(e)ine, methionine, arginine, histidine and lysine contents of protein preparations from senescent leaves of *Trifolium subterraneum*, expressed as percentages of the protein N occurring in these forms, were found to be 5·39–5·71, 2·80–2·90, 1·65–1·85 (best 1·80), 1·42–1·47, 1·02–1·13, 13·5, 4·26 and 5·72, respectively.

2. From comparison of these values with those previously found for non-senescent material (arginine, histidine and lysine values were not available) and from other considerations, it has been concluded tentatively that the onset of senescence was accompanied by an increase in the cyst(e)ine and possibly in the tyrosine content, and a decrease in the methionine content of the whole protein in the tissues.

We wish to thank Mr D. S. Riceman for providing the leaf material used in this work.

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Nicotinic Acid in Cereals

1. THE EFFECT OF GERMINATION

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Nutrition surveys have shown that under present conditions dietary supplies of nicotinic acid are being obtained largely from cereals. These vary widely in their nicotinic acid content. Maize and oats are poor sources, rice and barley are better and whole wheat is probably the best of the staple cereals.

Kodicek (1940) found that chemical methods gave higher results for maize than biological methods, and suggested that better agreement might be obtained if extraction were carried out with hot water instead of acid or alkali. Melnick (1942), reporting the results of a collaborative study, stated that with acid extraction chemical methods gave higher results than

microbiological assays for low-extraction wheat flour. With aqueous extraction to exhaustion lower results were obtained by both methods, the chemical still being higher than the microbiological. James, Norris & Wokes (1947) confirmed Melnick's findings with acid extraction for low-extraction flour, and extended them to maize and yeast, using a modified chemical method which gave satisfactory agreement with microbiological assays for whole wheat, barley and other foods. Various American workers (Oser, Melnick & Siegel, 1941; Cheldelin & Williams, 1942; Krehl & Strong, 1944) have reported the presence in cereals of so-called precursors of nicotinic acid which, although water-soluble, are inactive microbiologically. Hydrolysis with acid or alkali, or enzyme treatment converts these 'precursors' into microbiologically active substances. Other American workers (Burkholder & McVeigh, 1942; Burkholder, 1943; Davis, Laufer & Saletan, 1943) have shown that the nicotinic acid content of wheat, barley, oats and maize increases after germination, thus indicating the occurrence of a precursor possibly related to the so-called precursor of the previous workers. We thought it desirable to study by chemical as well as by microbiological methods the day-to-day changes in nicotinic acid content during germination, and thus obtain a more complete picture.

METHODS

Chemical assays

These were made by the method described by James *et al.* (1947). The coefficient of variation of a single assay averaged 3·6 in 310 consecutive assays on 97 samples of cereals and cereal preparations.

Microbiological assays

The method of assay using *Lactobacillus arabinosus* 17/5 as test organism, first described by Snell & Wright (1941), was later modified by Krehl, Strong & Elvehjem (1943) and by Barton-Wright (1944). The latter method, or that described in a Report to the Society of Public Analysts (1946), was used in the present work. Results have been calculated by reference to the equation for the standard curve, calculated by the method of least squares, or by comparison of the slopes of the similarly calculated standard and sample curves. The latter method (Wood, 1945, 1946) is to be preferred, but valid assays will give closely agreeing results by either method. In some instances the limits of accuracy were calculated by the elegant, but somewhat lengthy, method of Finney (1945).

In 364 assays on 31 samples (see Table 1) the average coefficient of variation of a single assay lay between 6 and 7%.

Germination methods

Laboratory scale. Selected seeds (50–75 g.) were planted in sterile sand in Petri dishes, moistened with distilled water and grown in the dark in a warm room or at 27°. At the end of the germination period (which was varied in different experiments) the seeds were washed free of sand with distilled water, and the degree of germination was determined by observing in each seed the ratio of the length of the shoot (acrospire) to the length of the seed, as in the method of the Association of Official Agricultural Chemists (1945), but using an extended scale (i.e. 1–2, 2–4, above 4) to cover the greater amount of germination obtained. The seeds were sorted into groups according to this scale of acrospire ratios and the percentage in each group was calculated. The seeds were then heated at 70–80° overnight and the average weight per seed was determined for different degrees of germination by weighing the whole of the seeds in each group. The seeds were then ground to no. 60 powder and the moisture content determined by heating at 105° for 3 hr. This was used to determine the average dry weight per seed.

Large scale 'stocking method'. The seeds were placed in calico bags containing 6–7 lb. each. These were first steeped at 10–12° for 1–4 days, according to the material, and then placed on the germinating floor under the same conditions as in the normal malting of barley. Malting was allowed to proceed as usual, records being kept of temperature and moisture additions. Each day one of the bags was removed to the kiln to check enzyme action. The kilned seeds were then sent to King's Langley. Here, weeds and other foreign matter, of which there was not more than 1%, were removed by hand. The given batch was sampled and the samples were examined in the same manner as the laboratory-germinated samples to determine the degree of germination. The average dry weight per seed in this batch was determined by weighing representative samples of 50–100 seeds. At least 9 such samples were taken. The coefficient of variation of the mean, calculated from the individual results on these 9 samples, lay between 1 and 2 (see Table 6). Before assay, a portion of the batch was ground to no. 60 powder, care being taken to ensure that the whole of the material passed through the sieve and was thoroughly mixed. This powdered material was stored in air-tight bottles and used for assay as soon as possible. Malted cereals were found to keep better in the unground condition.

Preparation of malt extracts

Since kilning had checked enzyme action without destroying the enzymes, the action of the latter could continue during the preparation of malt extract. In regard to barley, evidence had already been obtained (Klatzkin, Norris & Wokes, 1946) showing that in the brewing of malt extract more riboflavin might be produced. We therefore thought it of interest to examine this possibility for nicotinic acid. The malt extracts were prepared by methods ensuring the maximum yield of protein and aneurin (see Organ, Duncan, Westbrook & Wokes, 1943), since this might also be expected to ensure high yields of other B vitamins.

RESULTS

Barley

Large-scale experiments

Six typical batches of English barley were examined before and after large-scale malting, and the malt extracts made from the respective malted

barleys were also assayed. Mean results by the chemical method (see Table 2) showed no significant increases in nicotinic acid content at either stage.

Table 1. *Mean results by microbiological assay for the nicotinic acid content of samples*

B = Barley. *M* = Malted barley. *ME* = Malt extract.

Sample	Days germinated	No. of assays	Mean content (μg./g. dry wt.)
B 25	0	19	87 ±2·2*
M 25	12	15	103 ±1·9
ME 25	—	23	105 ±2·6
B 32	0	6	102 ±3·5
M 32	12	5	123 ±2·8
ME 32	—	6	114 ±2·2
B 35	0	19	109 ±2·2
M 35	12	23	117 ±2·5
ME 35	—	16	112 ±2·4
B 36	0	12	125 ±2·9
M 36	12	6	139 ±2·9
ME 36	—	6	122 ±1·4
B 38	0	15	115 ±2·7
M 38	12	19	121 ±3·0
ME 38	—	18	131 ±3·8
B 40	0	16	86 ±2·3
M 40	12	15	108 ±2·1
ME 40	—	19	128 ±2·7
Wheat	0	6	58·8±0·9
Wheat	10	6	62·8±0·8
Maize	0	19	31·4±0·4
Maize	1	6	34·6±0·7
Maize	2	4	36·8±0·8
Maize	3	5	31·9±1·1
Maize	6	4	27·3±0·75
Maize	9	4	26·6±0·45
Maize	12	16	33·8±0·4
Oats	0	8	11·8±0·27
Oats	4	6	17·0±0·33
Oats	8	8	19·8±0·56
Oats	12	14	21·0±0·28

* Standard error of mean.

Table 2. *Changes in nicotinic acid content of barley during malting and brewing, as shown by chemical assays*

Series no.	Nicotinic acid (μg./g. dry wt.) Barley	Malted barley	Malt extract	Increase during Malting (%)	Brewing (%)
25	99	104	106	5·1	2·0
32	111	116	116	4·5	0·0
35	123	112	125	−8·9	10·7
36	123	129	121	4·9	−6·5
38	127	126	122	−0·9	−3·2
40	112	120	122	7·1	0·9
Mean	116	118	119	1·9	0·4

Mean results by the microbiological method (see Table 1) showed an average increase of 14·7% during malting, but no significant increase during the brewing of the malt extract. Each of the six samples showed, microbiologically, an increase during malting and in one sample this exceeded 25%. There was thus a divergence between the chemical and microbiological results. A comparison of these (Table 3) showed that, with malted barley and malt extract, the average results by the two methods were in good agreement, and the individual deviations were scarcely significant when compared with the respective coefficients of variation. On the other hand, with unmalted barley the mean chemical result was 12% higher than the mean microbiological result. In five out of six samples the chemical method gave appreciably higher results, and in at least one of these (series 40) the difference was highly significant ($p = 0{\cdot}99$). Dr E. R. Dawson also kindly assayed this sample, barley 40, by the microbiological method and obtained a mean result of 90 μg./g. dry weight, confirming our microbiological result. With barley, therefore, the chemical method may give results appreciably higher than the microbiological method.

Table 3. *Comparison of chemical and microbiological results*

Series no.	Chemical results as % of microbiological results Barley	Malted barley	Malt extract	Coefficient of variation of mean chemical result Barley	Malted barley	Malt extract
25	114	101	101	4·5	0·03	1·2
32	109	94	102	7·4	7·8	4·9
35	113	91	112	4·6	3·6	4·9
36	98	93	99	2·0	4·6	4·1
38	110	104	93	3·9	3·5	5·1
40	130	111	97	1·6	5·9	5·3
Mean	112	99	101	—	—	—

These data summarize results obtained in 170 assays. At least 4 assays were made on each sample.

The results of microbiological assays indicate an apparent increase in nicotinic acid content when barley is malted. However, seeds lose weight on germination—the so-called malting loss—and this must be taken into account in assessing the true change in vitamin content. We therefore determined the average dry weight of a barley seed before and after malting. The results showed an average loss of 12–13% in weight, sufficient to account for almost the whole of the observed increase in nicotinic acid content. Hence, any nicotinic acid which had been formed from a precursor must have been used up in metabolic processes.

Small-scale experiments

In these, the barley was kept at 27° for 10 days, a sample from the material which was malted on the large scale being used. Acrospire counts showed that in some of the barley the degree of germination was considerably higher than in normal malting, the

shoot being longer than the seed in 33 % of the seeds, as compared with less than 1 % in normal malting. Nevertheless, no significant increases in nicotinic acid content were obtained.

Wheat

A large-scale experiment was carried out in which 1 cwt. of English wheat was germinated at 14–20° for 12 days. Perhaps owing to the warm weather, there was some development of mould after 10 days on the floor. The experiment was discontinued at that point, when the chemical results (Table 4) showed a significant increase of 33 % in the nicotinic acid content, after allowance for the loss in weight of the seeds. Microbiological assays on the initial and final samples showed no increase, the average content of a single seed being 2·30 μg. before germination and 2·27 μg. after germination (both calculated on dry weight). On the ungerminated wheat the chemical result was 108 % of the microbiological. On the germinated wheat the chemical result was 147 % of the microbiological result, a significant difference.

Table 4. *Changes in dry weight and nicotinic acid content during germination of wheat*

Stage of malting		Nicotinic acid content by chemical method						
		Individual assays (μg./g. dry wt.)					(μg./seed)	
Days steeping	Days on floor					Mean	Actual	As % of initial
0	0	64·5	56·6	65·7	65·6	63·8	2·46	100
2	6	68·2	67·5	—	—	67·9	2·46	100
2	0	85·3	84·9	83·5	82·9	84·2	2·82	115
2	10	92·3	91·6	—	—	92·0	3·24	132

Maize

A large-scale experiment was carried out with 1 cwt. of maize (White Horse Tooth variety) germinated at 15–18° for 12 days. Acrospire counts and weight changes showed that a fair degree of germination had occurred, but there was no increase in nicotinic acid content when allowance was made for changes in dry weight. The chemical method showed a net loss of 6 % after 1 day on the germinating floor, and of 10 % after 12 days on the floor. The microbiological method showed no significant change. The chemical results were 107–116 % of the microbiological results.

A small-scale experiment was carried out on the same sample in a warm room at 16–22° and also showed no increase in nicotinic acid content, although the acrospire counts indicated that a considerable degree of germination had occurred, the percentages of seeds with different acrospire ratios being as follows: 0–0·5, 9 %; 0·5–1, 56 %; 1–2, 15 %; 2–4, 13 %; above 4, 7 %. After 8 days' germination there was a net loss of about 12 %, and after 10 days a net loss of about 15 % of nicotinic acid.

Rice

For this, we used paddy containing the husk. The small quantity available permitted of only small-scale experiments at 27°. In 9 days' germination the average net increase in nicotinic acid content was 17 % (Table 5). Of the seeds 93 % had germinated, but germination was rather uneven. Of the germinated seeds, 52 % had an acrospire ratio greater than 1·0. There was a much greater increase in nicotinic acid content in the seeds showing the greater degree of germination. In 7 days' germination 84 % of the seeds had germinated and the increase in

Table 5. *Changes in dry weight and in nicotinic acid content during germination of rice (paddy) as shown by chemical assays*

Period of germination (days)	Acrospire ratio	Nicotinic acid content				
		Individual assays (μg./g. dry wt.)			(μg./seed)	
				Mean	Actual	As % of initial
0	—	74·1	73·0	73·6	1·88	100
7	1–4 (12 %)	95·2	100·0	97·6	2·41	128
9	Below 1 (48 %)	79·3	82·4	80·9	1·85	98
9	1–2 (33 %)	96·0	95·3	95·7	2·30	122
9	Above 2 (19 %)	124·0	116·4	120·2	2·85	152

Figures in parentheses represent percentage of total quantity of germinated rice which had reached the stated degree of germination.

seeds with acrospire ratios above 1·0 was 28% as compared with 33% for the corresponding degree of germination after 9 days. So far as we are aware, this is the first time that a significant increase in nicotinic acid content has been shown to occur in rice as the result of germination. It would seem worth while to carry out experiments on a larger scale when more material becomes available.

Oats

A large-scale experiment was carried out in which 1 cwt. of English oats was germinated at temperatures ranging from 15 to 18° for 12 days. The results (Table 6) showed by both chemical and microbiological methods almost the same percentage increase above the respective initial values. The average nicotinic acid content/seed by the microbiological method was, however, only about two-thirds of that determined chemically for the same sample. The differences between the chemical and microbiological figures were all highly significant ($p = 0{\cdot}99$). Each of the chemical figures in Table 6 is the mean obtained from at least four assays. The coefficients of variation of the mean (as calculated from the respective standard deviations of the mean) for the figures for samples 1–13 ranged from 0·2 to 3·7 and averaged 1·4. For sample 14 there was a higher coefficient of variation of 8·5 obtained from seven assays. We cannot explain why the results for this sample showed this wider deviation. However, the nicotinic acid content/seed for sample 14 falls into line with the nicotinic acid content found in the other samples. The rate of increase in nicotinic acid content of an average seed in the latter part of the experiment had slowed down considerably since the rapid initial increase.

Acrospire counts on the samples in the large-scale experiment showed only a moderate degree of germination. We therefore carried out on the same batch of oats a small-scale experiment, aiming at a higher degree of germination by using a temperature of 27°. Acrospire counts showed that this had in fact occurred. After 10 days' germination, the chemical method showed a net increase of 108% compared with 43% in the large-scale experiment.

DISCUSSION

American workers using microbiological methods first showed that germination of cereals may, under certain conditions, increase their nicotinic acid content. Our own experience leads us to suggest that in further investigations it would be desirable to relate any observed increases to the degree of germination, as determined from acrospire counts and weight changes. This might explain some of the discrepancies between the findings of different workers and also help to build up a more complete picture of the germination process. For barley, e.g., Burkholder (1943) obtained with a single sample an increase of 70% in nicotinic acid content/g. dry weight. When allowance was made for loss in weight during germination about one third of this apparent increase disappeared. We did not obtain a significant net increase with any one of our six samples in assays either by chemical or by microbiological methods. Moreover, there was still no

Table 6. *Changes in nicotinic acid content and average weight/seed during germination of oats*

			Nicotinic acid content				
			Chemical method			Microbiological method	
				(μg./seed)		(μg./seed)	
Sample no.	Treatment	Average dry weight of a seed (mg.)	(μg./g. dry wt.)	Actual	As % of initial	Actual	As % of initial
1	Untreated	35·3±0·41*	19·5	0·69	100 ±0·3*	0·42	100
2	Steeped 24 hr.	32·0±0·79	23·0	0·74	107 ±3·2	—	—
3	Steeped + 1 day on floor	32·8±0·54	26·9	0·88	127·5±0·6	—	—
4	Steeped + 2 days on floor	35·7±0·50	27·2	0·97	141 ±5·2	—	—
5	Steeped + 3 days on floor	32·9±0·31	30·7	1·01	146 ±0·4	—	—
6	Steeped + 4 days on floor	29·5±0·37	28·3	0·83	123 ±0·2	0·50	119
8	Steeped + 6 days on floor	32·1±0·28	27·8	0·89	129 ±1·7	—	—
10	Steeped + 8 days on floor	30·2±0·47	32·8	0·99	143 ±1·1	0·60	143
12	Steeped + 10 days on floor	30·7±0·54	34·9	1·07	155 ±3·1	—	—
13	Steeped + 11 days on floor	30·8±0·44	30·8	0·95	138 ±1·7	—	—
14	Steeped + 12 days on floor	31·5±0·64	34·4	1·08	157 ±13·4	0·66	157

* Standard error of mean.

The nicotinic acid content for each sample is the mean of at least 4 assays, of which the extreme range in the initial sample was 98–104 and in the final sample 92–108% of the respective mean.

significant increase when we prepared malt extracts from the malted barleys and thus gave still further scope to the germination enzymes. Our findings agree with those of Davis *et al.* (1943) who obtained an average increase of about 11 % in seven samples of barley as the result of germination, but made no allowance for loss in weight during germination, which would probably explain the whole of their observed increase.

With wheat, Davis *et al.* (1943) obtained an average increase of about 15 % in five samples. Our results indicate that the loss in weight during germination of wheat seeds (Fig. 1) might easily reach 15 %. If allowance were made for this loss in weight, the results of Davis *et al.* would probably not show any significant increase. Burkholder (1943) obtained a net increase of 24 % with a single sample. We obtained a net increase of 33 % by the chemical method, but no increase microbiologically.

Maize is of particular interest because of its low initial content of nicotinic acid, which has been held responsible for the prevalence of pellagra in parts of the world where it is a staple food. Burkholder & McVeigh (1942) obtained an increase of 141 %, uncorrected for loss in weight during germination. Burkholder (1943), after making such correction, obtained a net increase of 258 % on another sample. We did not obtain, either by chemical or microbiological methods, any increase in a sample with an initial content of 35·9 μg./g. dry matter. This may have been due to the high initial content as compared with 17 and 9·5 μg./g. dry matter respectively in the samples of the American workers. American findings indicate that greater increases may be expected with cereals of lower initial nicotinic acid content. Our results, on the whole, support this suggestion, but rice was an exception, since, using a high degree of germination, we secured an increase of 52 % in a sample with an initial content of 74 μg./g. The mean increase in the whole sample after 9 days' germination was only 17 %. In the work above our chemical assays had given the same general picture as our microbiological assays, and thus provided additional evidence of the reliability of the chemical method (cf. James *et. al.* 1947).

Oats, however, told a different story. Our chemical results for oats ungerminated and at different stages of germination were consistently 60–70 % higher than the microbiological values, though both methods showed the same net increase (57 % of the respective initial values) as the result of germination. This increase was considerably lower than the net increase of 352 % obtained by Burkholder (1943) microbiologically with one sample of oats. He, however, used oats with a much lower initial value and also obtained a much higher degree of germination. Fig. 1 shows the loss in weight at different stages of germination in our various samples, the data being used to calculate the net changes in nicotinic acid content recorded in our tables. The discrepancy between the chemical and the microbiological results was clearly established by the results of numerous assays, and will require further investigation.

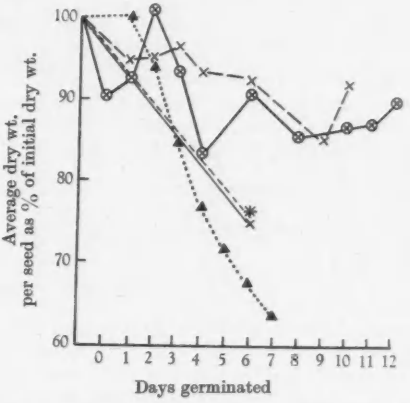

Fig. 1. Changes in dry weight of seeds during germination. All seeds in our experiments were steeped in cold water for 24 hr. before germination. Results obtained on wheat by Burkholder (1943) ×——×, Klatzkin, Norris & Wokes (present study) ×— — —×; on oats by Burkholder (1943) ✱ - - - - ✱, McVeigh (1944) ▲· · · · ▲, Klatzkin, Norris & Wokes (present study) ⊗——⊗.

SUMMARY

1. Samples of barley, maize, rice (paddy) and oats have been germinated in the laboratory under known conditions. Large-scale germination experiments have been carried out on samples of barley, wheat, maize and oats. The degree of germination was followed by acrospire counts and by changes in dry weight. Nicotinic acid assays have been made on the dried powdered material, both by a chemical and a microbiological method, at different stages of germination.

2. In oats a net increase was obtained of 57–108 %. Rice gave net increases of 0–52 % with different degrees of germination. With wheat, barley and maize no significant increase was obtained.

3. It is suggested that the considerably greater increases reported by American workers may be due to their obtaining a higher degree of germination, and to some extent also to their omitting to allow for changes in weight during germination.

4. The chemical method gave satisfactory agreement with the microbiological method with all samples, except the wheat and oats and one sample of unmalted barley, where the chemical method gave higher results.

We are indebted to the Curator of the Imperial Institute, South Kensington, for a sample of Burma paddy, to Samuel Thompson and Sons Ltd. for carrying out large-scale malting of our samples of barley, wheat, oats and maize, and to Miss J. Horsford and Mrs R. Wallach for assistance. The work undertaken by one of us (C.K.) in this investigation forms part of the work for a Ph.D. thesis in the University of London.

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A Polarographic Study of some Stilbene Derivatives of Biological Interest

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4-Aminostilbene and some related compounds have been shown to have an inhibitory action on the growth of young rats and on malignant tumours (Haddow, Kon & Harris, 1945). Certain of these substances are also potent carcinogens capable of eliciting tumours at sites remote from that of administration (Haddow, Kon & Harris, 1946). The ethylenic bond present in stilbene has been found to be electroreducible at the dropping mercury electrode (Laitinen & Wawzonek, 1942) and the present investigation was undertaken for the purpose of comparing the polarographic behaviour of compounds of this series showing differing biological activities. It was also considered that the data obtained in such experiments would be of value in assessing the suitability of the polarographic method of analysis in metabolic studies. Since stilboestrol is also a stilbene derivative (4:4′-dihydroxy-$\alpha\beta$-diethylstilbene), and similar problems arise in connexion with investigations of its metabolism in the human subject (Warren, Goulden & Robinson, 1948), it was decided to include synthetic oestrogens of this type in the investigation.

EXPERIMENTAL

Materials

All the substances used in these experiments were pure compounds. A number of the stilbene derivatives containing basic substituents was kindly put at our disposal by Prof. G. A. R. Kon and Dr R. J. C. Harris. We are also greatly indebted to Prof. E. C. Dodds and Dr W. Lawson for generous gifts of stilboestrol derivatives.

Solvent and electrolyte

The solvent employed was a dioxan-water mixture (3 : 1 by vol.) (Laitinen & Wawzonek, 1942). Commercial dioxan (British Drug Houses Ltd. 'purified') was refluxed for 12 hr. with sodium and then fractionated. Repetition of the sodium treatment was sometimes necessary in order to achieve very negative decomposition potentials. Only recently purified dioxan was used since there was some deterioration on storage.

Laitinen & Wawzonek recommend the use of tetrabutylammonium iodide as a supporting electrolyte for the attainment of very negative discharge potentials. In the present experiments tetraethylammonium iodide was found to be quite suitable, and it was easily prepared by heating

together triethylamine and ethyl iodide in absolute ethanol on a steam bath. The quaternary salt is very sparingly soluble in ethanol and rapidly separates. The salt was collected and recrystallized from absolute ethanol. It is stable and not deliquescent. The base solution was a 0·1 M-solution of this electrolyte.

Polarograms

The current-voltage curves were recorded on a Tinsley ink-recording polarograph. Control curves were first recorded using the base solution alone. The curve for the base solution containing the substance under examination was then superimposed on the record of the control curve. In all the measurements the observed diffusion currents were corrected for the residual current given by the base solution. O_2 was removed from the solutions by flushing with a current of H_2. The anode was a Hg pool of relatively large area and its potential was measured against a saturated calomel electrode (S.C.E.). The anodic potential was reproducible at −0·41 V. All cathodic potentials are also referred to the S.C.E.

The dropping Hg electrode had the following characteristics. At a pressure of 71·0 cm. of Hg the drop time (t) in the electrolyte solution used was 3·05 sec. (open circuit). The Hg flow (m) was 1·96 mg./sec. The calculated value of $m^{\frac{2}{3}} t^{\frac{1}{6}}$ is 1·887. The value of m was determined by direct weighing up to a negative potential of 2·4 V., and was found to be constant over this range. Direct measurements of t were also made over the same range, but the short drop times occurring at potentials more negative than 2·0 V. were checked independently by measurements of the diffusion current of nitrobenzene over the range −1·4 to −2·4 V. The effect of the decreased drop time reduced the product $m^{\frac{2}{3}} t^{\frac{1}{6}}$ from a value of 1·837 at −1·4 V. (t = 2·6) to 1·566 at −2·4 V. (t = 1·0), and the observed diffusion current of nitrobenzene showed a similar linear decrease over this range. All measurements were carried out at 25°.

RESULTS

In Table 1 the results obtained with stilbene derivatives not bearing substituents on the ethylenic carbon atoms are shown. Table 2 contains the measurements on compounds containing substituents in these positions. For each compound the range of concentration over which measurements were made is given together with the half-wave potential $E_{\frac{1}{2}}$ (*v.* S.C.E.), and the observed diffusion current i_d, after correction for the residual current. The half-wave potentials (in the tables negative signs have been omitted) have not been corrected for iR drop; with the type of cell used this correction would be small. The diffusion current shown by a mM-solution has been calculated from the measurements made at each concentration. The essential constancy of the half-wave potential with varying concentration of the substance, in those compounds that permitted its measurement, indicates that the electrode process is independent of the concentration of hydrogen ions, and justifies the use of unbuffered solutions in these measurements.

DISCUSSION

Two of the compounds included in this investigation have been examined by other workers (Laitinen & Wawzonek, 1942). For stilbene they reported $E_{\frac{1}{2}} = -2{\cdot}14$ V. (*v.* S.C.E.) and $i_d/C = 4{\cdot}04$. The corresponding values for this substance from Table 1 are −2·17 and 4·12. Although the half-wave potential determined in the present experiments agrees with that found by Laitinen & Wawzonek, the agreement between the values for i_d/C is less good when allowance is made for the difference in capillary characteristics. At the voltage used by Laitinen & Wawzonek for measurement of the diffusion current of stilbene (−2·31 V.), $m^{\frac{2}{3}} t^{\frac{1}{6}}$ had a value of 1·828 in their experiments, while the corresponding value in the present experiments was 1·593. If correction is made for the difference between the two factors the value of i_d/C shown in Table 1 becomes 4·73 as compared with the value of 4·04 given by Laitinen & Wawzonek.

The same workers also measured triphenylethylene and their values of $E_{\frac{1}{2}} = -2{\cdot}12$ and $i_d/C = 3{\cdot}55$ may be compared with the data in Table 2. Again agreement between values for the half-wave potentials is good, but the value for i_d/C found in the present experiments is higher (3·86) and, when corrected for difference in capillary, considerably higher (4·31) than that found by the American workers.

The half-wave potentials of most of the compounds shown in Table 1 fall within a narrow range. In the stilbene series, substitution in one or both nuclei results in a slightly more negative half-wave potential. The substituted stilbene analogues containing a naphthalene nucleus (last four compounds in Table 1), however, give waves at potentials equal to that of stilbene or more positive. This is particularly striking in the case of α-4-dimethylaminophenyl-β-2′-naphthylethylene. The second wave shown by these substances at approximately −2·50 V. is presumably due to reduction of the naphthalene nucleus, since reduction of this hydrocarbon gives a wave at this potential.

The general similarity of the values of i_d/C for most of the compounds in Table 1 indicates that there are no large differences in diffusion coefficients among these substances. In the cases of 4-amino-4′-hydroxystilbene and 4-amino-4′-hydroxy-2′-methylstilbene, which show rather lower values for i_d/C, it seems probable that these are due to changes in the nature of the potential-determining reaction at the dropping electrode rather than to reductions in diffusion rates. From the recorded current-voltage curves, plots were made of $\log i/(i_d - i)$ against voltage. In general the reciprocal slopes of these graphs were in approximate agreement with the theoretical value of 0·059 V.

for a reversible, potential-determining reaction involving one electron. However, the plots of the data for 4-amino-4′-hydroxystilbene and for 4-amino-4′-hydroxy-2′-methylstilbene gave slopes of 0·11 V. in the 4 and 4′ positions are very susceptible to oxidation and it is possible that small quantities of such oxidation products were present in the specimens used.

Table 1. *Polarographic data for stilbene derivatives not bearing substituents on the ethylenic carbon atoms*

Compound	$-E_{\frac{1}{2}}$ (V. *v.* S.C.E.)	i_d (μamp.)	C (mM)	i_d/C
Stilbene	2·17	2·70	0·655	4·12
	2·17	5·40	1·310	4·12
4-Aminostilbene	2·30	2·12	0·646	3·28
	2·32	4·42	1·292	3·42
	2·31	6·68	1·938	3·44
4-Methoxystilbene	2·24	1·48	0·398	3·72
	2·24	3·04	0·796	3·82
	2·25	4·39	1·194	3·78
4-Hydroxystilbene	2·34	1·50	0·476	3·15
	2·34	2·27	0·714	3·18
	2·34	2·94	0·952	3·09
4-Dimethylaminostilbene	2·29	2·18	0·588	3·71
	2·29	4·39	1·176	3·73
	2·29	6·41	1·764	3·64
3-Dimethylaminostilbene	2·19	1·62	0·453	3·57
	2·19	3·29	0·906	3·63
	2·19	4·81	1·359	3·54
4-Amino-4′-hydroxystilbene	2·45	1·03	0·446	2·31
	2·44	2·00	0·892	2·24
	2·46	3·02	1·338	2·26
4:4′-Dimethoxystilbene	2·33	1·19	0·320	3·72
	2·31	2·34	0·640	3·66
	2·32	3·53	0·960	3·68
4-Dimethylamino-2′-methylstilbene	2·32	1·76	0·518	3·40
	2·30	3·60	1·036	3·48
	2·30	5·49	1·554	3·53
4-Dimethylamino-3′-methylstilbene	2·34	1·60	0·554	2·89
	2·33	3·68	1·108	3·32
	2·33	5·56	1·662	3·34
4-Dimethylamino-4′-methylstilbene	2·36	1·87	0·565	3·31
	2·35	3·67	1·130	3·25
	2·34	5·70	1·695	3·36
4-Dimethylamino-2′:5′-diethylstilbene	2·37	0·90	0·303	2·97
	2·33	1·90	0·606	3·14
	2·34	4·43	1·515	2·92
4-Amino-4′-hydroxy-2′-methylstilbene	2·41	1·74	0·724	2·40
	2·44	2·49	1·086	2·30
2:4:6:2′:4′:6′-Hexamethylstilbene	2·47	1·08	0·752	1·44
	2·48	2·48	1·880	1·32
α-4-Dimethylaminophenyl-β-1′-naphthylethylene	2·12 2·50	1·32	0·322	4·10
	2·12 2·50	2·70	0·644	4·19
	2·10 2·50	3·94	0·966	4·08
α-4-Dimethylaminophenyl-β-2′-naphthylethylene	1·80 2·49	0·87	0·188	4·63
	1·82 2·49	1·90	0·470	4·06
α-4-Dimethylaminophenyl-β-1′-(2′-methyl)-naphthylethylene	2·18	0·73	0·239	3·05
	2·17	1·60	0·478	3·34
	2·17 2·54	3·75	1·195	3·14
α-Phenyl-β-1-(4-dimethylamino)naphthylethylene	2·08 2·53	—	—	—

Although the nature of the available data precluded a high degree of accuracy in plotting these derived curves, there seems little doubt that the electrode process differed in these compounds. Stilbene derivatives bearing both amino and hydroxyl groups

Certain of the compounds in Table 1 are of especial interest since they possess carcinogenic properties. 4-Aminostilbene, 4-dimethylaminostilbene, 4-dimethylamino-2′-methylstilbene and α-4-dimethylaminophenyl-β-1′-naphthylethylene have all been

reported as inducing malignant tumours in rats (Haddow *et al.* 1945, 1946). From the data of Table 1 there is nothing to distinguish such biologically active substances from an inactive one such as stilbene.

with the waves developed by anthracene, naphthalene, and phenanthrene under the same conditions.

In general, the polynuclear aromatic hydrocarbon carcinogens give two waves at approximately −1·90 and −2·50 V. These waves are characteristic for the

Table 2. *Polarographic data for stilbene derivatives bearing substituents on the ethylenic carbon atoms*

Compound	$-E_{\frac{1}{2}}$ (V. *v.* S.C.E.)	i_d (μamp.)	C (mM)	i_d/C
α-Ethylstilbene	2·30	1·57	0·408	3·84
	2·31	3·14	0·816	3·84
	2·31	4·65	1·224	3·80
αβ-Diethylstilbene (m.p. 71·5–72·5°)	*NW*	—	—	—
αβ-Diethylstilbene (m.p. 74–75°)	(2·59)	—	—	—
Stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene)	*NW*	—	—	—
Dienoestrol (3:4-di-(4′-hydroxyphenyl)hexa-2:4-diene)	*NW*	—	—	—
4:4′-Dihydroxy-α-methyl-β-ethylstilbene	*NW*	—	—	—
4:4′-Dihydroxy-α-methyl-β-cetylstilbene	*NW*	—	—	—
4:4′-Dihydroxy-α-phenyl-β-ethylstilbene	*NW*	—	—	—
2:2′-Dihydroxy-αβ-diethylstilbene	*NW*	—	—	—
4-Methoxy-αβ-diethylstilbene	*NW*	—	—	—
4:4′-Diacetyl-αβ-diethylstilbene	1·77	1·34	0·172	7·80
	1·78	2·71	0·345	7·85
	1·79	4·07	0·517	7·85
4:4′-Dihydroxy-α-*n*-propylstilbene	2·52	2·80	1·890	1·47
4:4′-Dihydroxy-α-*n*-amylstilbene	2·53	1·80	1·570	1·15
4:4′-Dihydroxy-α-*iso*propylstilbene	(2·61)	—	—	—
Triphenylethylene	2·12	1·22	0·328	3·72
	2·09	2·53	0·656	3·86
	2·10	3·80	0·984	3·86
Triphenylmethylethylene	2·24	1·20	0·330	3·64
	2·25	2·45	0·660	3·72
	2·24	3·59	0·990	3·63
α-4-Hydroxyphenylstilbene	2·17	0·86	0·266	3·23
	2·17	1·75	0·532	3·29
	2·17	2·68	0·798	3·35
α-Phenyl-ββ-di-4-hydroxyphenylethylene	2·28	1·04	0·320	3·25
	2·29	2·28	0·640	3·56
α-Ethyl-β-4-hydroxyphenylstilbene	2·36	2·20	0·467	4·72

Compounds showing no reduction at the dropping electrode are indicated by *NW* (no wave). Two compounds appeared to give indications of waves at very negative potentials and these values are included in parentheses.

It is interesting to compare the data for these substances with those for other carcinogens. Wawzonek & Laitinen (1942) showed that, although aromatic compounds possessing a high degree of resonance, such as benzene, are not reduced at the dropping mercury electrode, aromatic polynuclear hydrocarbons showing a diminished degree of resonance are reducible. In experiments which have been confirmed and extended by the present authors, they established the polarographic behaviour of such carcinogens as 3:4-benzpyrene, methylcholanthrene, 1:2:5:6-dibenzanthracene, and 1:2-benzanthracene. The polarographic waves resulting from reduction of these compounds in aqueous dioxan were compared

reduction of an anthracenic structure (9 : 10 addition) followed by that of the resulting naphthalenic nucleus (1 : 4 addition). The polarographic reduction resembled, in the relative ease and site of attack, such reactions as the addition of alkali metals or reduction by ethanol and alkali metals.

From the data in Table 1 it is clear that the susceptibility of the ethylenic bond of stilbene derivatives to reactions involving addition at this site is intermediate between that shown by the anthracenic and naphthalenic bonds of the hydrocarbon carcinogens. Possibly, with stilbene derivatives, as with polycyclic aromatic hydrocarbons, this degree of susceptibility is an important feature in their

adsorption on to enzymes. From a comparative point of view the stilbene ethylenic bond is analogous to the 1 : 2 double bond in pyrene ($E_{\frac{1}{2}} = -2{\cdot}10$) rather than to the 9 : 10 double bond in phenanthrene ($E_{\frac{1}{2}} = -2{\cdot}44$).

All but one of the compounds shown in Table 2 are α- or αβ-substituted stilbene derivatives. The exception is dienoestrol in which there is a conjugated hexadiene system. In general, monosubstitution on the α-carbon atom results in reducible derivatives with half-wave potentials approximately the same or slightly higher than that of stilbene itself. This applies to both α-alkyl and α-aryl derivatives. The nature of the substituent groups (alkyl or aryl) is, however, of decisive importance in disubstituted derivatives. Stilbene derivatives with alkyl groups on both α and β carbon atoms are, in general, not reducible at the mercury cathode. In the exceptional case of αβ-diethylstilbene, two specimens were available; one had m.p. 71·5–72·5°, the other m.p. 74–75°. These are probably the *trans* and *cis* forms, respectively, of this hydrocarbon (Goulden, unpublished). Although the constitution of these two substances has not yet been finally established, the evidence at present available suggests that the *trans* form is not reducible. The higher-melting form gave some evidence of a very ill-defined wave at approximately −2·6 V. Apart from this one rather doubtful exception, αβ-dialkyl derivatives of the stilbene and stilboestrol series were not reducible. The large wave shown by 4:4′-diacetyl-αβ-diethylstilbene is presumably produced by reduction of the keto groups, since reduction of acetophenone under the same conditions gave a wave at about the same potential (−1·67 V.). Disubstitution with one alkyl and one aryl group produces reducible derivatives, e.g. triphenylmethylethylene and α-ethyl-β-(4-hydroxyphenyl)-stilbene.

One object of the present work was to explore the possibility of using polarographic methods of analysis in the detection and estimation of stilbene derivatives in metabolic experiments. The results indicate at least one serious practical difficulty. The half-wave potentials lie between about −2·2 and −2·6 V., i.e. more negative than the discharge potentials of ions of the alkali metals or of ammonium (*c.* −2·2 V.). The presence of small amounts of these ions makes it impossible to detect or measure the reduction of stilbene derivatives. Preliminary experiments on blood and urine from rats treated with 4-dimethylaminostilbene showed that the preparation of extracts suitable for polarographic measurements (i.e. free from Na^+, K^+ and NH_4^+) was difficult, and in no case was a satisfactory measurement obtained.

SUMMARY

1. Polarographic data are presented on 37 compounds of the stilbene and stilboestrol series.

2. Although certain of the stilbene derivatives possessed carcinogenic potency, no differentiation could be made between these compounds and related non-carcinogenic stilbenes.

3. Stilbene derivatives of the types, α-alkyl, α-aryl, or α-alkyl-β-aryl, are reducible at the dropping mercury electrode. Derivatives of the type αβ-dialkyl are not reducible.

4. αβ-Dialkylstilboestrols and dienoestrol are not reducible.

5. The highly negative half-wave potentials of stilbene derivatives make the use of polarographic methods difficult for the detection and estimation of these substances in biological materials.

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An Electrophoretic Study of Diphtheria Toxoid Preparations

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It has been found that batches of diphtheria toxoid produced in these laboratories for the immunization of horses exhibit varying degrees of antigenicity. This variation has not been correlated with any variations in the constitution of the medium used for the toxin production or in the conditions of growth of the organism thereon. Since it would be of considerable advantage to possess a method of rapidly assessing the probable antigenic quality of a toxoid preparation prior to its injection into horses, it was thought that an investigation of the electrophoretic behaviour of different batches of toxoid would be worth while, as providing a possible criterion of antigenic potentialities.

Although the results of these investigations did not reveal any consistent differences among groups of toxoid preparations which were known to produce good and poor antigenic responses, a number of points arose which are worth recording. A considerable amount of work has been carried out on the electrophoretic behaviour of the corresponding antibodies (cf. Kekwick & Record, 1941), but data referring to diphtheria antigens are confined to observations by Lundgren, Pappenheimer & Williams (1939), who published a short account of the electrophoretic behaviour of highly purified diphtheria toxin; they did not extend their work to toxoid.

EXPERIMENTAL

Production and preparation of toxoids. Most of the toxoids were prepared from toxins produced on a papain digest of beef medium, except for one produced on a tryptic digest of beef, and another on an acid hydrolysate of casein medium. These preparations had to be concentrated 40–50 times to provide a solution strong enough for the investigation. Concentration was carried out by means of an ultrafilter, and, as dialysis against buffer caused some loss of toxoid through the membrane, the toxoid was thoroughly washed on the ultrafilter with the requisite buffer solution until the refractive indices of the washings and of fresh buffer were indistinguishable within the limits of error of the dipping refractometer. The refractive index of the toxoid preparation was measured at this stage, and dilution with buffer made to a standard difference of 0·00400 between the refractive index n of the protein solution, and n_0 that of the buffer. Mobility measurements were made in solutions of half this strength. The buffer usually employed was a phosphate buffer (pH 8·0, ionic strength $\mu = 0{\cdot}1$). Buffers used in mobility determinations were phosphate buffers, $\mu = 0{\cdot}1$, except for pH values below 6, where acetate buffers were necessary, and at values above 8, where borate buffers were used.

Electrophoretic technique. Runs were carried out in the Tiselius (1937) apparatus at +0·5°. The potential gradient in the **U** tube was held constant, usually at some value in the range 1·5–3·5 V./cm. The diagonal *schlieren* method (Philpot, 1938) was employed, using as light source a mercury vapour lamp from which monochromatic light ($\lambda = 546$ mμ.) was isolated by means of a filter. The curves were photographed on Kodak Half-Tone (Wratten) Panchromatic Plates (*P* 25).

Measurements of pH were made with a double hydrogen electrode, using 0·1 N-HCl (pH 1·075) as the standard reference solution. Conductivity measurements on the buffers, necessary for mobility calculations, were made in a Washburn-type conductivity cell, and the distances moved by the components were measured from enlargements (of known magnification) obtained by tracing on graph paper the outlines of the patterns projected from the plates in a photographic enlarger.

A few runs, for the purpose of investigating different fractions, were carried out in the large type of **U** tube as employed by Blix, Tiselius & Svensson (1941).

RESULTS

Electrophoretic patterns of various diphtheria toxoid preparations

Typical curves are shown in Fig. 1 *A–F*. These indicate generally two major components, present in rather different relative amounts in the different preparations. A quantitative estimate was not attempted since the separation of the two peaks could not be satisfactorily achieved for this purpose. Examination of the contours of the curves also reveals that, in some cases, these components are themselves undoubtedly composite (e.g. see Fig. 1 *A*, *B*). Another feature, most prominent in Fig. 1 *E*, *F*, is a sharp spike projecting from the stationary ϵ boundary (for the origin of the ϵ boundary see Longsworth & MacInnes, 1939). At one period there was a suggestion that antigenicity might be associated with the material responsible for this peak, but an extension of the observations to a larger number of toxoid preparations (about 30) showed that this could not be correct. The correlation of electrophoretic patterns with the antigenic response in horses was further complicated by the large variation in the responses of different horses to the same batch of toxoid.

The curves shown in Fig. 1 were all obtained from toxoids produced on papain digest media.

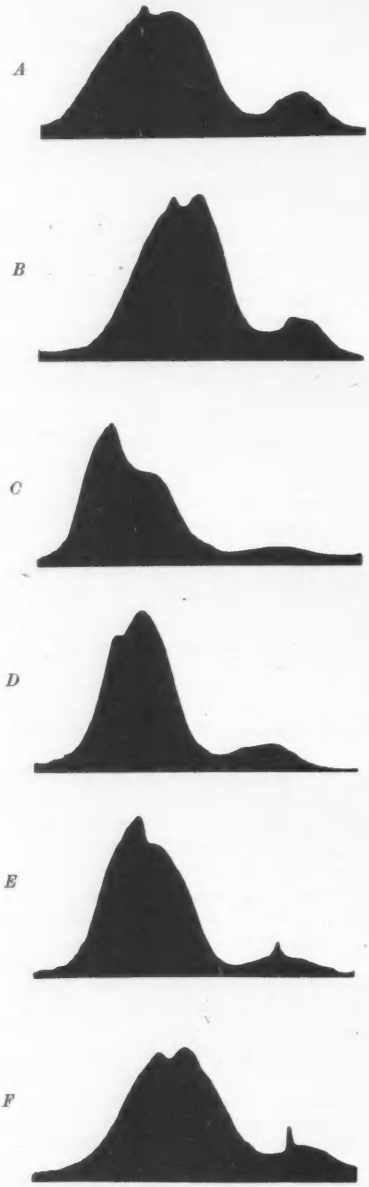

Fig. 1. Electrophoretic patterns of typical diphtheria toxoid preparations (see text) in phosphate buffer pH 8, $\mu = 0{\cdot}1$, 2·6 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. All these toxoids were produced from a papain digest of beef medium.

Fig. 2 shows the patterns *A* and *B*, obtained from toxoids produced on an acid hydrolysate of casein and a tryptic digest of beef medium respectively. The latter shows the presence of a new component. Methods of purification similar to those described by Pope & Linggood (1939) and Linggood (1939)

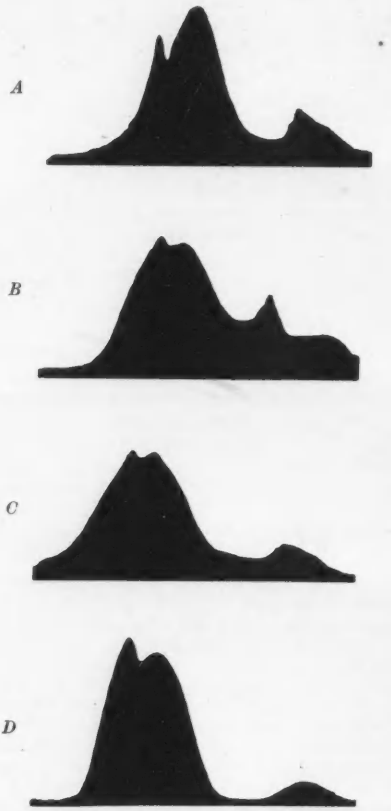

Fig. 2. Electrophoretic patterns of diphtheria toxoid preparations in phosphate buffer pH 8, $\mu = 0{\cdot}1$, 2·8 V./cm. Exposures approximately 2 hr. after starting current. Only cathode limb curves are recorded. Migration is from right to left. Details of these preparations are mentioned in the text.

were attempted. Treatment of the tryptic digest toxoid with 5% Farnell's *L.S.* carbon (Farnell Carbons Ltd., 1A Conduit Road, S.E. 18) removed most of this new component (see Fig. 2*C*). Treatment with 7% Farnell's *L.S.* carbon followed by precipitation from the filtrate by addition of 15% (v/v) of 5% (w/v) cadmium chloride solution, and subsequent extraction from the precipitate by 2% (w/v)

$Na_2HPO_4 . 12H_2O$ solution gave the product shown in Fig. 2*D*. The components here appeared to be purer, judging by the regular contours of the peaks.

Treatment of ordinary (papain digest) toxoids with 5% Farnell's *L.S.* or Sutcliffe, Speakman's *P.Z.* charcoal (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) showed that the electrophoretic pattern was scarcely affected.

Ammonium sulphate fractionation of concentrated toxoid produced no significant alteration in the relative amounts of the components present, either in the filtrate or in the precipitate.

A method of purification developed by Dr C. G. Pope, to be described elsewhere, produced a significant alteration in the electrophoretic pattern. A sample of toxoid purified by this method and assaying at 1700 *Lf* doses in each mg. of protein nitrogen gave, at pH 5·5, the pattern shown in Fig. 3. It consists of approximately 80% of slow-moving material together with smaller amounts of faster-moving impurities. This pattern should be compared with that given by an untreated toxoid at the same pH (see Fig. 5).

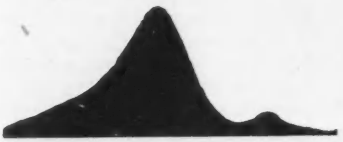

Fig. 3. Electrophoretic pattern of a purified diphtheria toxoid preparation in acetate buffer pH 5·5, 3·4 V./cm. Exposure 199 min. after starting current. The cathode limb is shown. Migration is from right to left.

Investigation of electrophoretically separated fractions

Runs were carried out in both the ordinary and the large **U** tube, to obtain, if possible, fractions representing one or other of the components present in toxoids. Isolation of fractions was carried out by hydrostatic displacement of the boundaries to the requisite position followed by sectioning off the **U** tube compartments which could be subsequently emptied independently. First, as regards the material responsible for the small, sharp, nearly stationary peak in some toxoids (e.g. see Fig. 1*E*) solutions containing only this component were isolated, analyzed for total nitrogen, and tested for activity by flocculation. Samples from other sections of the **U** tube were also tested as a check that toxoid activity had not been greatly diminished during the course of the experiments. The strongest solution of the minor component thus obtained gave total N 40·8 μg./ml. (allowing for a blank on the buffer), but no *Lf* units could be detected. Thus, this component could not possess activity of more than about 25 *Lf* units/mg. N and would therefore be able to make only a very small contribution, if any, to the *Lf* units present.

In a similar manner solutions containing the fastest component were isolated and examined. Again, when care was taken to ensure that this component was obtained free from others, no toxoid activity was detectable in it by flocculation testing, although solutions containing nitrogen equivalent to 0·5–1·0% protein were isolated. It would thus appear that the toxoid activity is associated with the slower of the main components.

To test this conclusion a mixture of a toxoid with an equivalent amount of antitoxin (known, from previous electrophoretic work, to contain only β and γ globulins) was flocculated. The floccules were spun off and the supernatant liquid, concentrated and washed with buffer on an ultrafilter, then submitted to electrophoresis. The pattern obtained showed that most of the residual protein consisted of the inactive portion of β, γ globulins; but there was also some material which corresponded with the faster major component of the toxoid. This result was in accord with the experiments just described.

Mobility measurements. Mobility measurements were made over the range of pH values (5·0–9·0) throughout which the toxoids are fairly stable. Calculations were made only for the major components, and only from descending boundary rates (see Longsworth & MacInnes, 1940). Values of the mobilities for both components varied slightly from one preparation to another. Typical results are shown in Fig. 4. Each point is the mean of two determinations on the same solution. The curves can be extrapolated to indicate that the isoelectric points are approximately in the region of pH 3·5–4·0, as is known from other data.

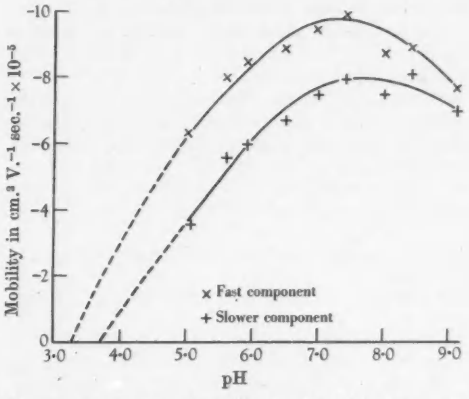

Fig. 4. Electrophoretic mobilities of diphtheria toxoid components in the pH range 5·0–9·0. Values are uncorrected for buffer viscosities.

Below a pH of about 6 there was a greater tendency for the composite nature of the peaks to be emphasized; thus at pH 5·5 the slower major peak can be seen to possess a very distinct hump, indicative of a boundary due to material which is moving rather more slowly (see Fig. 5).

Fig. 5. Electrophoretic pattern of a diphtheria toxoid preparation in acetate buffer pH 5·5, 1·7 V./cm. Exposure 335 min. after starting current. The cathode limb is shown. Migration is from right to left.

DISCUSSION

Some discussion of the sharp peak appearing in the electrophoretic pattern of certain toxoid preparations appears to be called for. It is somewhat reminiscent of effects produced by convection and of the β-anomaly (Longsworth, Shedlovsky & MacInnes, 1939). Reasons for assuming that it is not due to convection are (*a*) the detection of non-dialyzable nitrogen in this boundary, (*b*) the reproducible nature of the phenomenon with a given toxoid. Thus it was found that when a particular toxoid was divided into separate samples and submitted to electrophoresis, the curves obtained either all failed to exhibit this peak or all showed it; and, when present, it was in the same position and moved at the same slow rate. Repeated washing with buffer, on the ultrafilter, did not cause this peak to vanish from the pattern of any toxoid which originally showed it.

An unusual feature is the shape of the mobility curves, the speed of migration attaining a maximum value in each case at about pH 7·0–8·0. Although the mobilities are uncorrected for buffer viscosities, the corrections would not cause differences large enough to remove the effect of these maxima. A similar effect has recently been observed by Watson, Cromartie, Bloom, Heckly, McGhee & Weissman (1947) in the case of glutamyl polypeptide, etc. However, in their work the effect appeared only in the results obtained from the anode (ascending) limb, which is known to give anomalous results (Longsworth & MacInnes, 1940). It appears highly probable that the causes of the maxima are similar in the two cases, due to interaction between the molecules of the substance under examination, or to interaction with the buffer.

SUMMARY

1. Electrophoretic patterns of diphtheria toxoid preparations have been obtained, and typical curves are shown.

2. Most preparations show two major peaks with, in some cases, a slow-moving minor peak. The components represented by the major peaks, judging by their contours, are not quite homogeneous.

3. One preparation, purified chemically, appeared to be about 80% pure.

4. Toxoid activity has been assigned entirely, or almost entirely, to the slower major component.

5. The mobilities of the two major components have been measured over a pH range of 5·0–9·0.

6. Attempts to correlate the electrophoretic pattern with antigenicity have so far failed.

The author wishes to express his thanks to Dr F. V. Linggood and to Dr C. G. Pope of these laboratories, the former for suggesting this investigation, and the latter for discussions during the course of the work. He is also indebted to Mr E. Fenton for technical assistance in the preparation of the materials for electrophoresis.

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New Zealand Fish Oils

4. OBSERVATIONS ON THE OIL CONTENT OF FRESH WATER EELS

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(*Received* 25 *August* 1947)

Two species of fresh water eel *Anguilla australis schmidtii* Phillips and *A. dieffenbachii* Gray are known to occur in New Zealand, where they are conveniently termed respectively, short-finned and long-finned eels. Although the former species has been comparatively neglected apart from the determination of the vitamin A and vitamin D content of the body oil (Denz & Shorland, 1934; Cunningham, 1935; Weeber, 1945), the latter species has received the attention of several investigators in respect of the fatty-acid composition of the body oil (Shorland & McIntosh, 1936) and distribution of vitamin A and oil in the tissues (Edisbury, Lovern & Morton, 1937). The contributions made by the latter investigators as well as by Lovern (1938) on the European eel, *A. anguilla* L., suggest that the oil content increases with the length of the fish. McCance (1944), however, denied that the oil content could be foretold from the length, and his data showed that the oil content of the migrant eel, irrespective of length, was relatively constant. In regard to the immature eels it is our belief that no generalization was justified as he used only two samples and, although our results agree with those of McCance in respect of migrant eels, the results for immature eels support Lovern's (1938) contention that the oil content increases with the length of the eel.

EXPERIMENTAL

The material used in this work was collected from Lake Ellesmere migrant populations in 1942 and from immature eels from that lake in 1943 and 1944 as well as from Foxton Lakes, 1943, Manawatu River, 1943, and Hutt River, 1943. To eliminate the possibility of seasonal effects the samples were taken during the months of March and April. The eels were divided into length groups, each group from each locality containing usually 5–10 eels commencing with the length group 22 in. and under, and proceeding by 3 and 4 in. intervals respectively for short-finned and long-finned eels, up to the largest sizes obtained. This yielded four groups of short-finned eels and four to six groups of immature long-finned eels. The migrant long-finned eels were in general longer than the immature specimens, and were divided into four groups, covering a range of 39–55 in. General consideration of the length-weight relationships will be published elsewhere, but it may be stated here that the same species of eel showed similar relationships in the various localities studied.

The eels from each group were subdivided into head, trunk, tail, liver, viscera (excluding liver) and ovary, when the latter was present. The head was severed at the first vertebra, while the trunk and tail portions were separated by a transverse cut at the vent.

RESULTS

The proportions of the various tissues in the eels examined are shown in Table 1.

In both short-finned and long-finned species, the immature eels from Foxton Lakes are shown to have heads which are significantly larger than those from other localities studied. Other locality differences of a lesser degree may also be noted in the proportions of other tissues of the immature eels. Comparing migrant with immature eels it will be seen that the changes in tissue proportions are confined in the case

Table 1. *Proportions of the dissected tissues of eels expressed as a percentage of the total weight*

Source	Head	Trunk	Tail	Ovary	Viscera	Liver
			Short-finned eels			
†Lake Ellesmere, 1943	4·79±0·61*	43·19±1·52	45·93±0·32	—	5·16±0·78	0·93±0·05
†Lake Ellesmere, 1944	5·04±0·50	43·49±0·62	45·67±0·77	—	5·08±0·80	0·72±0·04
†Foxton Lakes, 1943	9·84±1·38	41·91±1·19	43·59±1·36	—	4·11±0·50	0·55±0·02
†Hutt River, 1943	4·07±0·27	41·91±0·76	49·38±1·28	—	3·55±0·37	1·09±0·06
‡Lake Ellesmere, 1942	4·8 ±0·43	43·30±1·17	45·60±0·90	3·5	*c.* 1·83	0·97±0·07
			Long-finned eels			
†Lake Ellesmere, 1943	9·08±1·01	46·05±0·61	37·02±1·18	0·64	6·17±0·34	1·04±0·01
†Lake Ellesmere, 1944	7·42±1·16	44·79±0·89	41·79±2·11	—	5·24±0·46	0·76±0·06
†Foxton Lakes, 1943	12·96±0·24	42·16±1·37	39·12±1·38	—	4·78±0·39	0·98±0·18
†Hutt River, 1943	7·42±0·87	42·72±0·88	43·17±1·60	—	5·51±0·43	1·18±0·06
†Manawatu River system, 1943	10·18±0·38	39·12±1·72	44·42±2·23	—	5·26±0·46	1·02±0·05
‡Lake Ellesmere, 1942	5·5 ±0·4	43·2 ±1·28	36·9 ±1·26	10·7±0·8	2·0 ±0·31	1·7 ±0·05

* Standard error. † Immature eels. ‡ Migrant eels.

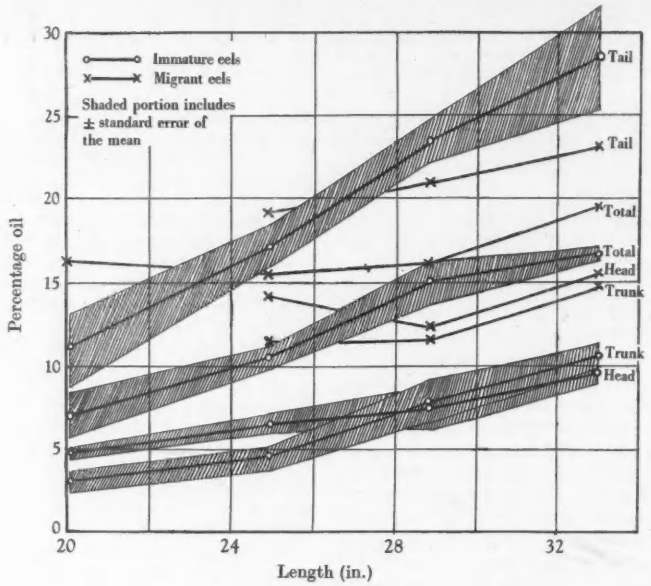

Fig. 1. Oil content of tissues of *A. australis*.

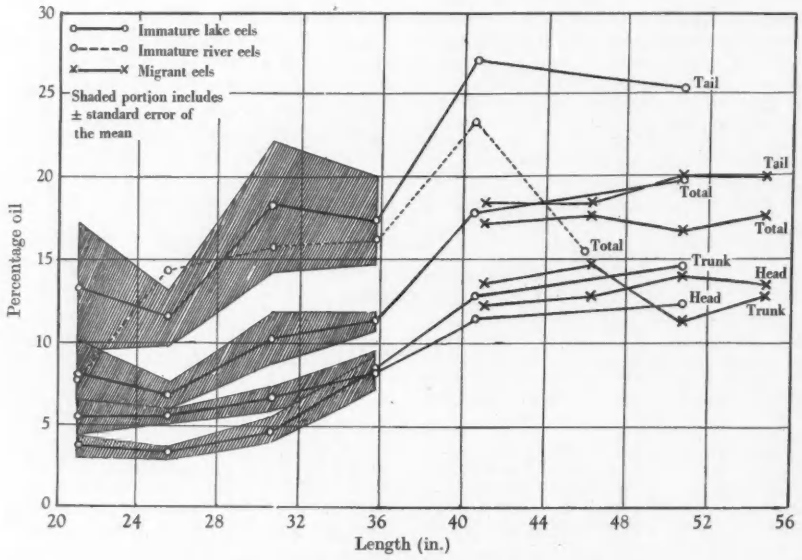

Fig. 2. Oil content of tissues of *A. dieffenbachii*.

of the short-finned eels to the development of 3·5 % of ovary with a corresponding reduction in the amount of viscera. In the long-finned species the transition from the immature eel to the migrant is accompanied by a relatively greater production of ovary (10·7 %) and a significant increase in the proportion of liver, with a decrease in the amount of head.

The oil content of the various tissues of the eels is given in Figs. 1 and 2. As no significant differences were observed between the oil content of similar groups of short-finned eels from different localities the separate results for the percentage total oil and percentage oil in the dissected tissues have been respectively combined. For similar reasons results for long-finned eels from Foxton Lakes and Lake Ellesmere have also been combined, while the average total oil content of the Manawatu and Hutt River specimens, which were significantly oilier, are shown separately. Since, however, the results for the oil content of the tissues of the long-finned eels from the Manawatu and Hutt Rivers showed similar variations to those exhibited by the Foxton Lakes and Lake Ellesmere specimens, they have been omitted from Fig. 2.

It will be observed from Figs. 1 and 2 that the oil content of immature eels tended to increase with the length of the fish in agreement with Lovern's (1938) observations on *A. anguilla* L. This tendency, however, appeared to be confined to the immature eel, the migrant showing little, if any, increase in oil content with length.

The general picture of the relationship between the oil content and length of both species is shown to be somewhat similar, but the long-finned eels may be distinguished from the short-finned by their greater variability in the oil content of the tail as well as by their less uniform relationship between total oil content and length. Attention is drawn to the interesting relationship between the oil content of the head and trunk which was found to be similar in both species. In the smaller immature specimens the oil content of the head exceeds that of the trunk, but with the larger immature specimens the reverse is true. In the migrant eel the situation in regard to the relative oil content of the head and trunk is shown to revert back to that of the smaller immature eel.

Certain differences may be noted in respect of the oil content of the two species. The short-finned migrant is shown to contain (cf. Fig. 1) a higher percentage of total oil than the immature eel of the same length, while in the case of the long-finned species (cf. Fig. 2) the reverse is true. In the short-finned species the migrant, as compared with the immature eel of the same length, tended to have a lower percentage of oil in the tail but a higher percentage in the head and trunk. In this connexion the long-finned species resembled the short-finned as regards the tail oil, but differed in respect of the trunk and head oil percentages which showed relatively small variation between migrant and immature specimens of the same length (Table 2).

Table 2. $\frac{\text{Percentage of oil in tail}}{\text{Percentage of oil in head}}\left(\frac{T}{H}\right)$ and $\frac{\text{Percentage of oil in tail}}{\text{Percentage of oil in trunk}}\left(\frac{T}{F}\right)$ *in immature and migrant eels*

	Immature eels		Migrants	
	T/F	T/H	T/F	T/H
Short-finned	3·42 ± 1·06*	2·80 ± 0·84	1·69 ± 0·13	1·51 ± 0·19
Long-finned	3·29 ± 1·13	2·54 ± 0·85	1·47 ± 0·20	1·47 ± 0·06

* Standard deviation.

In considering the ratios T/F and T/H in Table 2, immature long-finned specimens of over 38 in. have been excluded, as such specimens may contain appreciable proportions of ovary. The ratios T/F and T/H show a much greater uniformity in the migrant eels as compared with immature eels, as well as a more uniform distribution of oil in the former as compared with the latter. The results shown in Figs. 1 and 2 are consistent with the hypothesis that greater uniformity in the distribution of oil in the migrant, as compared with the immature eel, is achieved by means of a considerable movement of oil from the tail to the trunk, followed by a further movement of oil from the trunk to the head. In the long-finned species, however, the situation is complicated by the development of the ovary as an important oil reserve (cf. Fig. 1), and it is suggested that, comparing long-finned species with short-finned, the relatively low proportion of oil in the trunk and head sections of the migrant as compared with the immature long-finned eel is due to a further considerable diversion of the trunk oil into the ovary.

In Figs. 3 and 4 the oil contents of the tissues are expressed as a percentage of the total oil reserves and compared with the proportions of these tissues in the fish, which, in the case of the short-finned eels were not significantly different as between migrant and immature eels, apart from the replacement in the former of the viscera by ovary. As no significant differences in oil distributions were found between groups of long-finned eels below 38 in. used in this work, the results for these groups have been combined as shown in Fig. 4.

In the immature short-finned eel, the tail, comprising 46 % of the total weight, contained 75 % of the total oil in the fish. With the onset of maturity the amount of oil in the tail was reduced to 57 % of the total oil reserves. In the immature long-finned eel the tail portion amounted to 41 % of the total weight and contained 68 % of the total oil reserves. At maturity the tail was slightly reduced in amount to 37 % with a spectacular reduction in oil content to 41 % of the total oil reserves, coinciding with the appearance of the ovary as an important oil depot, which accounted for 20 % of the total oil reserves in the fish as compared with only 7 % in the mature short-finned eel. Although the proportion of trunk (42–43 %) in both the short-finned and long-finned eels did not change as the fish matured, the oil reserves of this section of the fish, expressed as a percentage of the total oil reserves, increased respectively from 19 to 32 % and from 23 to 33 %.

SUMMARY

1. It was found that commencing with eels of *c.* 50 cm. length and proceeding to fully adult migrant eels the oil content of the immature eels tended to increase continuously with length from approximately 7 to 17 % in *A. australis* and from 7 to 23 % in *A. dieffenbachii*.

2. The largest size of immature eel was found to approximate in oil content to that of the migrant eel, the oil content of which was not found to vary appreciably with the length of the fish.

3. Considering the tissues separately, however, it was found that whereas the distribution of oil was fairly uniform in the migrant eel as between the head, trunk and tail, this was not so in the case of the immature eel which contained approximately 70 % of the total oil reserves in the tail comprising but 42–43 % of the total weight of the fish.

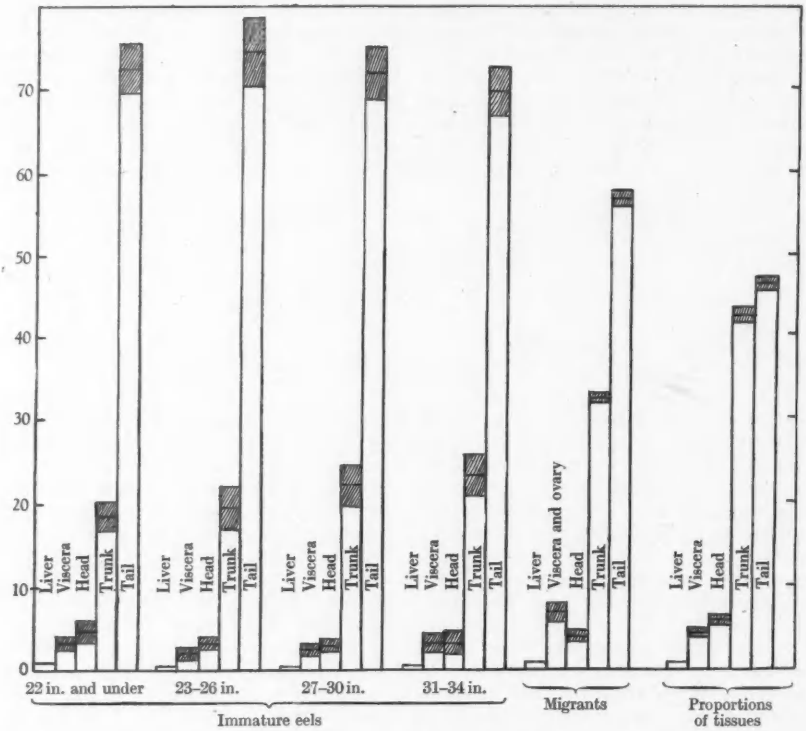

Fig. 3. Distribution of oil in the tissues of *A. australis* expressed as a percentage of the total oil. (Shaded portions include ± standard error.)

4. The observed results are consistent with the theory now put forward that just prior to migration a more uniform distribution of oil is attained by a partial transference of oil from the tail into the trunk and from thence into the head and ovary.

We wish to thank Mr A. E. Hefford, M.Sc., formerly Chief Inspector of Fisheries and Mr D. F. Hobbs, Senior Fresh Water Biologist to the Marine Department for encouragement and for generous assistance in the collection of samples.

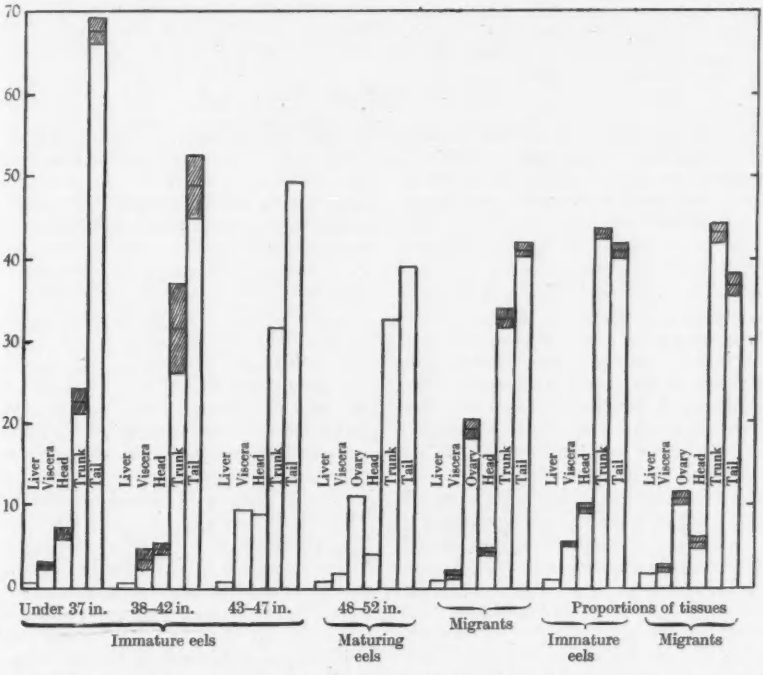

Fig. 4. Distribution of oil in the tissues of *A. dieffenbachii* expressed as a percentage of the total oil. (Shaded portions include ± standard error of the mean.)

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The Fate of Certain Organic Acids and Amides in the Rabbit

4. THE AMINOBENZOIC ACIDS AND THEIR AMIDES

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Investigations of the metabolism of the aminobenzoic acids in mammals have suggested that the principal methods by which these compounds are conjugated *in vivo* are acetylation of the amino group and conjugation of the carboxyl group with glucuronic acid. For example, Muenzen, Cerecedo & Sherwin (1926) found that *m*- and *p*-aminobenzoic acids, but not anthranilic acid, were acetylated by man and the rabbit, although none of the acids was conjugated in this way by the dog. Acetylation of *p*-aminobenzoic acid has also been observed in the rabbit by Ellinger & Hensel (1914), Hensel (1914), and Harrow, Mazur & Sherwin (1933) and in man and the guinea pig by Zehender (1943). Mitsuba & Ichihara (1927) reported that anthranilic acid was the only aminobenzoic acid to cause an increased excretion of reducing substances in the dog or rabbit, although Quick (1932) showed that in the dog all three isomers were conjugated to a considerable extent with glucuronic acid.

Other methods of conjugation are, however, recorded in the literature. Mitsuba & Ichihara (1927) found that anthranilic acid, but not the *m*- or *p*-isomers, gave rise to an increased excretion of ethereal sulphate by the dog and rabbit. Without satisfactory evidence they attributed this increase to the formation of salicylic acid. Salkowski (1882) also observed no increase in ethereal sulphate excretion when *m*-aminobenzoic acid was given to dogs or rabbits and claimed to have isolated some *m*-aminohippuric acid from the urine. He also stated that some *m*-ureidobenzoic acid (*N*-phenylurea-3-carboxylic acid) was formed. Hildebrandt (1903) claimed that this type of conjugation occurred with all three isomers in the rabbit and mouse, and Mitsuba & Ichihara (1927) confirmed this for anthranilic acid in the dog and rabbit. Muenzen *et al.* (1926) however, denied the formation of ureido- and aminohippuric acids in man, dog or rabbit although they gave little detail to support their statement. Formation of other ureido compounds *in vivo* has been suggested by early workers, e.g. from taurine in man and dog (Salkowski, 1872, 1873) and from sulphanilic acid in the dog (Ville, 1892); but Schmidt & Allen (1920) were unable to detect the excretion of ureidotaurine as a metabolic end product of taurine in man, and we have not obtained *p*-ureidobenzenesulphonic acid as a metabolite of sulphanilic acid in the rabbit (unpublished). In the present work, although we did not deliberately search for ureido acids, we have not made any observation which suggests their formation. Several workers have recorded that the aminobenzoic acids are partly excreted unchanged, e.g. Salkowski (1882) (*m*-isomer), Hildebrandt (1903) (all isomers), Muenzen *et al.* (1926) (*o*-isomer only in man and rabbit, all isomers in dog).

The metabolism of the aminobenzamides has received little attention. The hypothesis (Woods, 1940; Fildes, 1940) that the bacteriostatic effect of sulphanilamide is due to its structural similarity to *p*-aminobenzoic acid which enables it to compete with that compound in certain essential metabolic processes in bacteria led to the expected observation (Hirsch, 1942) that *p*-aminobenzamide had similar bacteriostatic activity to that of sulphanilamide. In the work reported here we have compared the fate of the aminobenzamides in the rabbit with that of their respective acids. The extent of conjugation with acetic, glucuronic and sulphuric acids and the excretion of ether-soluble acid show that, unlike the carbamyl group in benzamide (Bray, Neale & Thorpe, 1946*a*), the same group in aminobenzamides is comparatively stable to hydrolysis. One conspicuous difference between the acids and amides is that the latter are oxidized to aminohydroxybenzamides which are at least to some extent conjugated with sulphuric acid, whereas the acids are not oxidized, the principal conjugates excreted being ester-type glucuronides. Some of these metabolites (or derivatives of them) have been isolated and identified. The aminohydroxybenzoic acids isolated* support the view put forward in an earlier paper (Bray, Ryman & Thorpe, 1947) that when oxidation

* All oxidation products isolated in this work were acids. It is not known whether the actual compounds excreted are acids or amides. It is very probable that in the case of the *o*-isomer the amide is excreted, since the percentage oxidation is greater than the estimated degree of hydrolysis, but analytical data for the other isomers do not allow any similar deductions to be made. In this paper the term 'oxidation product' may apply to either acid or amide.

of a disubstituted aromatic compound is effected by the introduction of a hydroxyl group into the ring, the resulting phenol has a 1:3:4-configuration, although the isolation of 3-hydroxyanthranilic acid as an oxidation product of *o*-aminobenzamide makes it clear that the hypothesis must be extended to include such compounds.

METHODS

Preparation of aminobenzamide. The amides used were prepared by reduction of the corresponding nitrobenzamides using $FeSO_4$ and NH_3 (Jacobs & Heidelberger, 1917). The nitroamides were prepared by the action of ammonia upon the acid chlorides.

Diet and feeding. The rabbits used were does of 2–3 kg. maintained on a constant diet of rabbit pellets (Bruce & Parkes, 1946), except in the quantitative study of *p*-aminobenzoic acid and amide when bran and oats were used (for details of administration of the diets see Bray *et al.* 1947). We have no reason to believe that the metabolism of these compounds differs in any essential way on the two diets, except for variations in the 'base line' values of some normal metabolites as described below.

Dosage. Owing to their toxicity, anthranilic acid and its amide were not given in doses exceeding 0·5 g./kg. The other acids and amides were not toxic at dose levels up to 1 g./kg. The compounds were administered by stomach tube, the acids being dissolved in $NaHCO_3$ solution and the amides suspended in water.

Estimation of ether-soluble acid. The method was essentially that previously described (Bray *et al.* 1946*a*). We found that all three acids could be quantitatively extracted from water or urine at pH 4. This pH was attained in the urines by adding to 10 ml. urine 10 ml. of a solution containing no ether-soluble material and made up by mixing 40 ml. M-Na_2HPO_4 with 52 ml. M-$NaHSO_4$.

Estimation of diazotizable material. This was carried out according to the method of Bratton & Marshall (1939) as referred to previously (Bray *et al.* 1946*b*). Eckert (1943) states that in blood filtrates quantitative estimation of *p*-aminobenzoic acid requires 15–20 min. for diazotization and 30–60 min. for coupling. With our solutions we did not find any modification of the Bratton-Marshall procedure necessary. With the *o*-acid and amide, however, it was necessary to leave the coupled solution 22 hr. in order to attain maximum colour development. Kohl & Flynn (1941) state that a diazotization procedure using 3 hr. for colour development gives low values for anthranilic acid after hydrolysis, owing to the formation of an undiazotizable anhydride, anthranil, when solutions are heated at an acid reaction. We found that if 22 hr. were allowed for colour development, solutions of anthranilic or acetylanthranilic acids, which had been subjected to the usual hydrolysis procedure, gave values within 3% of the theoretical.

Anthranilic acid gives rise to a much more intense colour than an equivalent amount of the corresponding amide. To match 5·8 mg. of the former 10 mg. of the latter are needed. The conditions of hydrolysis used (1 hr. with 0·2 N-HCl in a boiling water bath) are not sufficiently drastic to cause significant hydrolysis of the carbamyl group. It has already been reported (Bray, James, Ryman & Thorpe, 1948) that only 8% hydrolysis of the amide occurs on heating with N-HCl in a boiling water bath for 1 hr. Solutions of *o*-aminobenzamide and its *N*-acetyl derivative subjected to the ordinary hydrolysis procedure gave quantitatively accurate results when compared with a standard untreated *o*-aminobenzamide solution. An amide standard was, therefore, always used in determinations on urines obtained after administration of *o*-aminobenzamide. It will be seen later that some hydrolysis of the carbamyl group occurs *in vivo*. The values obtained for the percentage of *o*-aminobenzamide excreted with a free amino group (Table 2) will therefore appear higher than they actually are. The oxidation products isolated after acid hydrolysis following the administration of *o*-aminobenzamide, 3- and 5-hydroxyanthranilic acids, give no appreciable colour upon diazotization and coupling under our conditions of estimation. This probably applies also to their amides, which are likely to be the compounds actually excreted. Stronger solutions of these acids (100–200 mg./100 ml.) give a violet colour on diazotization and coupling (see p. 440).

The oxidation products isolated from *m*-aminobenzamide urine after acid hydrolysis likewise give no diazo reaction. The difference in colour intensity of the diazotized and coupled acid and amide is much less than with the *o*-isomer (9·4 mg. acid ≡ 10 mg. amide), but is sufficient to require the use of an amide standard.

The colours developed from *p*-aminobenzoic acid and its amide are of equal intensity, but an amide standard was actually used in experiments in which *p*-aminobenzamide was given. This amide is only oxidized to a very small extent and the oxidation product (isolated as acid) gives no diazo reaction.

Estimation of reducing substances. This was carried out as previously (Bray *et al.* 1946*b*). The method does not give reliable results for ether-type glucuronide excretion if the amount of ester-type glucuronide excreted is high, as in the case of the aminobenzoic acids. Further, oxidation products may interfere if they are themselves reducing. Since the aminohydroxybenzoic acids isolated after the administration of *o*- and *m*-aminobenzamides are strongly reducing, values for these amides are probably unreliable. Values obtained with the *p*-amide can be accepted since it is only oxidized to a very slight extent.

Results of estimations of ester-type glucuronides formed from the aminobenzoic acids are probably somewhat low. Pure *p*-aminobenzoylglucuronide (see p. 439) gave values about 9% lower than the theoretical.

Estimation of ethereal sulphate. This was carried out as previously, by the method of Folin (1905–6).

EXPERIMENTAL AND RESULTS

Quantitative studies

Ether-soluble acids. The ether-soluble acid of the normal urine of rabbits on the bran, oats and cabbage diet ranged from 200 to 450 mg./day (calculated as hippuric acid) and on the pellet diet from 400 to 700 mg. Average values were 325 and 574 mg. respectively. The results obtained after administration of aminobenzoic acids and amides (calculated as aminobenzoic acid) are summarized in Table 1.

It should be noted that the ether-soluble acid values obtained represent only acid unconjugated with glucuronic acid since the glucuronides are not extracted by ether. Thus, the difference between the ether-soluble acid values for

Table 1. *Excretion of ether-soluble material (calculated as aminobenzoic acid) by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as ether-soluble acid								
	o-Compound			*m*-Compound			*p*-Compound		
Dose (g./kg.)	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0·25	131	60	36	—	—	—	133	45	29
	135	61	27	—	—	—	125	29	10
	137	79	38	—	—	—	137	20	Nil
	144	69	31	—	—	—	139	26	10
0·5	131	29	4	99	49	10	133	42	28
	131	15	19	99	52	—	135	41	11
	135	33	28	117	59	16	137	30	6
	137	42	5	117	62	—	139	24	10
	137	52	10	139	43	10	—	—	—
	—	—	—	139	47	—	—	—	—
	144	18	26	142	47	10	—	—	—
	144	26	19	142	45	—	—	—	—
	144	38	Nil	—	—	—	—	—	—
1·0	—	—	—	99	55	7	133	49	19
	—	—	—	117	61	8	135	32	12
	—	—	—	117	—	9	137	47	10
	—	—	—	139	37	6	139	24	13
	—	—	—	139	46	—	—	—	—
	—	—	—	142	39	10	—	—	—
	—	—	—	142	50	—	—	—	—

acid and amide does not give a true idea of the hydrolysis of the carbamyl group, unless the fact that some of the acid is excreted as glucuronide is taken into account (cf. Table 3).

The nature of the ether-soluble acid was investigated in the case of *p*-aminobenzoic acid. The ether extract from the urine of six rabbits which had each received 2 g. of the acid yielded 4 g. pure *p*-aminobenzoic acid. No other metabolite, e.g. *p*-aminohippuric acid, was detected. Owing to the difficulty of obtaining and estimating *o*- and *m*-aminohippuric acids the ether-soluble acid from the *o*- and *m*-isomers was not examined. Quick (1932) was unable to devise any satisfactory procedure for estimation or identification of aminohippuric acids in urine. It is theoretically possible that the apparently low results for anthranilic acid, at 0·5 g./kg. dose level, may be due to the formation of a conjugate which is not estimated by the method used.

Diazotizable material. Table 2 shows the results obtained for excretion of diazotizable material after administration of the three aminobenzoic acids and their amides. The values obtained directly on the urine, and given under the heading 'free', represent the sum of free acid or amide plus acid conjugated as ester-type glucuronide, if present; other substances which might contribute are conjugates of the acids with glycine (aminohippuric acids) or *O*-conjugates of aminohydroxybenzoic acids or amides with sulphuric or glucuronic acids formed as a result of oxidation. The aminohydroxybenzoic acids which have been identified as oxidation products (and presumably their amides) do not diazotize and couple under the conditions of estimation. Whilst it is not definitely established that the *O*-conjugates diazotize and couple, it is possible that they do. We find, for example, that *o*-aminophenylglucuronide diazotizes and couples, although *o*-aminophenol itself does not. Available evidence (see Table 5) suggests that the contribution of *O*-conjugates to the total of 'free' diazotizable material would be of significant magnitude only in the case of *o*-aminobenzamide. The additional diazotizable material obtained after hydrolysis of the urine is given under the heading 'acetyl' and can be assumed to represent acetamidobenzoic acid or amide except in the case of *o*-aminobenzamide. Here hydrolysis of an *O*-conjugate could cause a decrease in diazo value large enough to mask an increase due to deacetylation. We actually found that the hydrolyzed values never exceeded the unhydrolyzed, but since we were unable to isolate any *o*-acetamidobenzamide we have recorded 'Nil' values in Table 2.

The fact that neither anthranilic acid nor its amide appear to be acetylated in the rabbit may account for the greater toxicity of these compounds as compared with the other isomers. The values obtained for the percentage of 'free' amide excreted are certainly high since some amide is hydrolyzed to acid which gives a more intense colour on diazotization and coupling. It is not possible to calculate the true values with accuracy without knowing whether *o*-aminohippuric acid is present, the full extent of amide hydrolysis (i.e. whether any *o*-aminobenzoylglucuronide has been formed), and the diazotization and coupling properties of the *O*-conjugates. Table 4 shows that the sulphate conjugation of *o*-aminobenzamide corresponds to 26–40% of the dose. This is presumably due to 3- and 5-hydroxyanthranilic acids or their amides. A further small amount of these compounds may be excreted as ether glucuronide.

In the case of *m*-aminobenzamide there is a clear indication that acetylation occurs. The values for free amide excreted can be taken as reasonably accurate since the difference in colour intensity of the diazotized and coupled

Table 2. *Excretion of diazotizable material, free and acetylated (calculated as aminobenzoic acid or amide) by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as diazotizable material														
	o-Compound					*m*-Compound					*p*-Compound				
		Acid		Amide			Acid		Amide			Acid		Amide	
Dose (g./kg.)	Rabbit no.	Free	Acetyl	Free	Acetyl	Rabbit no.	Free	Acetyl	Free	Acetyl	Rabbit no.	Free	Acetyl	Free	Acetyl
0·25	131	75	Nil	33	Nil	—	—	—	—	—	103	87	9	22	46
	131	67	Nil	—	—	—	—	—	—	—	103	62	9	25	76
	135	81	Nil	41	Nil	—	—	—	—	—	103	77	4	—	—
	137	69	—	36	Nil	—	—	—	—	—	113	81	21	20	64
	144	59	Nil	31	Nil	—	—	—	—	—	113	80	13	16	68
	—	—	—	—	—	—	—	—	—	—	113	—	—	15	73
	—	—	—	—	—	—	—	—	—	—	121	83	9	24	81
	—	—	—	—	—	—	—	—	—	—	121	67	14	23	78
	—	—	—	—	—	—	—	—	—	—	121	—	—	21	77
	—	—	—	—	—	—	—	—	—	—	125	73	16	19	60
	—	—	—	—	—	—	—	—	—	—	125	67	4	—	—
	—	—	—	—	—	—	—	—	—	—	133	—	—	14	81
	—	—	—	—	—	—	—	—	—	—	137	—	—	12	62
0·5	131	69	Nil	50	Nil	99	70	10	23	41	103	87	1	37	45
	131	—	—	45	Nil	99	85	12	27	35	103	67	15	28	85
	135	68	Nil	48	—	99	82	23	—	—	113	71	18	35	51
	137	75	Nil	48	—	99	92	9	—	—	113	75	2	28	45
	144	71	Nil	45	Nil	117	78	Nil	24	42	113	78	14	—	—
	144	67	Nil	39	Nil	117	80	14	—	—	121	62	7	28	58
	—	—	—	—	—	117	76	19	—	—	121	83	21	31	55
	—	—	—	—	—	139	83	13	52	10	121	72	14	—	—
	—	—	—	—	—	139	100	Nil	50	10	125	70	13	38	41
	—	—	—	—	—	139	94	4	—	—	125	76	17	—	—
	—	—	—	—	—	142	74	11	50	6	—	—	—	—	—
	—	—	—	—	—	142	86	7	52	9	—	—	—	—	—
	—	—	—	—	—	142	82	4	54	2	—	—	—	—	—
	—	—	—	—	—	142	—	—	37	14	—	—	—	—	—
1·0	—	—	—	—	—	99	96	4	38	42	103	44	16	36	24
	—	—	—	—	—	99	—	—	41	30	103	57	1	—	—
	—	—	—	—	—	117	90	9	30	43	103	49	Nil	—	—
	—	—	—	—	—	117	81	17	25	41	113	51	1	42	37
	—	—	—	—	—	139	100	Nil	66	6	113	41	3	—	—
	—	—	—	—	—	142	70	Nil	49	6	121	41	3	34	35
	—	—	—	—	—	142	79	2	48	5	121	53	2	—	—
	—	—	—	—	—	—	—	—	—	—	121	62	1	—	—
	—	—	—	—	—	—	—	—	—	—	125	61	5	39	38
	—	—	—	—	—	—	—	—	—	—	125	86	8	—	—

acid and amide is of the same order as the experimental error of the diazo method. As with the *o*-isomer, the metabolite formed by oxidation would probably not contribute to the diazotizable material. The amounts formed, however, are much smaller and will be of correspondingly less significance. The *m*-acid is acetylated to only a small extent. The oxidation product of *p*-aminobenzamide would also probably not contribute to the diazotizable material.

Table 2 also shows that different rabbits may have widely different powers of acetylation. Rabbits 139 and 142 are consistently poor acetylators compared with 135 and 137. It has been our experience that rabbits which are not good acetylators are much less resistant to toxic amino compounds.

Reducing substances. The average base line values were 209 mg./day on bran, oats and cabbage and 185 mg. on the pellet diet. The results obtained after administration of aminobenzoic acids are given in Table 3. It will be seen that there is evidence of conjugation for all three isomers. The glucuronide excretion after administration of amides could be reliably estimated only in the case of the *p*-amide which is oxidized to only a very slight extent. The formation of considerable amounts of reducing oxidation products from the *o*- and *m*-isomers invalidates the glucuronide estimation.

Ethereal sulphate. The average base line values for SO_3 were 21 mg./day on the bran, oats and cabbage diet and 41 mg. on the pellet diet. The results obtained after administration of aminobenzoic acids and amides are summarized in Table 4. Increases in ethereal sulphate excretion were observed only after giving the amides. We did not confirm the observation of Mitsuba & Ichihara (1927) that anthranilic acid causes an increase in ethereal sulphate excretion.

Table 3. *Excretion of reducing substances (calculated as glucuronic acid) by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as glucuronide (ester or ether type)							
	o-Compound		*m*-Compound		*p*-Compound			
							Amide	
Dose (g./kg.)	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	Rabbit no.	Acid (ester type)	(Ester type)	(Ether type)
0·25	131	44	—	—	103	—	0	0
	131	65	—	—	113	8	0	0
	144	43	—	—	113	24	—	—
	144	47	—	—	121	—	8	0
	—	—	—	—	125	—	6	0
0·5	131	54	99	4	103	19	9	0
	144	43	99	17	113	22	10	1
	144	62	117	7	121	30	1	1
	—	—	117	8	125	14	3	0
	—	—	117	8	125	13	—	—
	—	—	139	4	—	—	—	—
	—	—	142	7	—	—	—	—
1·0	—	—	99	6	103	—	6	0
	—	—	99	8	113	21	7	0
	—	—	117	6	121	14	3	0
	—	—	117	7	125	15	6	0
	—	—	117	7	—	—	—	—
	—	—	139	7	—	—	—	—
	—	—	139	8	—	—	—	—
	—	—	142	6	—	—	—	—
	—	—	142	7	—	—	—	—
	—	—	142	10	—	—	—	—

Table 4. *Excretion of ethereal sulphate by rabbits after administration of aminobenzoic acids and amides*

	Percentage of dose excreted as ethereal sulphate								
	o-Compound			*m*-Compound			*p*-Compound		
Dose (g./kg.)	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide	Rabbit no.	Acid	Amide
0·1	—	—	—	—	—	—	103	0	—
	—	—	—	—	—	—	113	0	—
0·25	131	0	26	—	—	—	103	—	2
	144	0	32	—	—	—	113	0	—
	—	—	—	—	—	—	121	0	—
0·5	131	0	33	—	—	—	125	0	2
	144	0	40	99	0	—	121	—	1
1·0	—	—	—	99	0	6	103	—	5
	—	—	—	99	0	7	113	—	2
	—	—	—	117	0	5	121	0	1
	—	—	—	142	0	7	125	—	1

Qualitative studies

Isolation of m- *and* p-*acetamidobenzamides*

In the case of *m*- and *p*-aminobenzamides, the *N*-acetyl derivatives could be isolated by continuous extraction with ether from the urine of rabbits to which the free amides had been administered by stomach tube (dose level 1 g./kg.). The material thus obtained was dissolved in water, treated with charcoal and recrystallized from water. The products obtained were identical with authentic specimens of *m*-acetamidobenzamide (m.p. 221°) and *p*-acetamidobenzamide (m.p. 274°), which were prepared by the action of acetic anhydride on the respective amides. This is direct evidence that in these compounds the carbamyl group is not completely hydrolyzed in the rabbit. Similar evidence in the case of *o*-aminobenzamide is provided by the fact that, although the acetyl derivative could not be isolated by ether extraction, the amide itself was. Table 2 shows that this amide is not acetylated *in vivo*.

Isolation of ester type glucuronides as metabolites of acids

The method of isolation was essentially as previously described (Bray *et al.* 1947). Since the compounds being isolated were ester glucuronides and consequently less stable than those of the ether type, the time taken during isolation was reduced to a minimum.

Table 5. *Excretion of metabolites of aminobenzoic acids and amides by the rabbit*

Dose (g./kg.)	Isomer	Compound	Percentage of dose excreted as: Ether soluble acid	Ester glucuronide	Free NH_2 derivative	Acetyl NH_2 derivative	Ethereal sulphate	Estimated average hydrolysis (%)
0·25	Ortho	Acid	60–79	43–65	59–81	0	0	33*
		Amide	27–38	—	31–41	0	26–32	
0·5		Acid	15–52	43–62	67–75	0	0	12*
		Amide	0–28	—	39–50	0	33–40	
0·5	Meta	Acid	43–62	4–17	70–100	0–23	0	12*
		Amide	10–16	—	23–52	2–42	—	
1·0		Acid	37–61	6–10	70–100	0–17	0	8*
		Amide	6–10	—	25–66	5–43	5–7	
0·25	Para	Acid	20–45	8–24	62–87	4–21	0	15
		Amide	0–29	0–8	12–25	46–81	2	
0·5		Acid	24–42	13–30	62–87	4–21	0	20
		Amide	6–28	1–10	28–38	41–85	1–2	
1·0		Acid	24–49	14–21	44–86	0–16	0	21
		Amide	10–19	3–7	34–42	24–38	1–5	

* These values are probably low, since no values are available for ester glucuronide.

p-*Aminobenzoylglucuronide.* The solution remaining after the removal of PbS was evaporated to small bulk at 50–60° under reduced pressure. On standing crystallization occurred. The red-brown solid was filtered off, dissolved in water, decolorized with charcoal and recrystallized from aqueous ethanol. The product formed white crystalline flakes (m.p. 193° with decomp.; $[\alpha]_D^{24°} - 5·5°$ in water). It strongly reduced Benedict's and Fehling's solutions, and appeared to be a *complex* of equimolecular proportions of *p*-aminobenzoic acid and *p*-aminobenzoylglucuronide. (Found: C, 52·7; H, 4·41; N, 5·82%; equiv. (by titration) 225; NH_2 (diazotizable), 6·45%; glucuronic acid, 40·5%. $C_{20}H_{22}O_{10}N_2$ requires C, 53·3; H, 4·88; N, 6·23%; equiv., 225; NH_2, 7·12%; glucuronic acid, 43·2%.) The yield was 10% of the dose.

Low values for glucuronic acid content were consistently obtained by the Shaffer-Hartmann method. This is probably due to the great lability of these compounds to heat. Pure preparations on warming even slightly, whether dry or in solution, rapidly developed a bright-red colour.

By continuous ether extraction of an aqueous solution of the complex, *p*-aminobenzoic acid was isolated and characterized. By careful evaporation of the remaining solution at a low temperature under reduced pressure, a substance crystallizing as fine white needles was obtained which, after recrystallization from aqueous ethanol, melted (with decomp.) at 208–210°. It was strongly reducing and showed $[\alpha]_D^{24°} - 12°$ (in water). It appeared to be p-*aminobenzoylglucuronide.* (Found: C, 49·6; H, 4·34; N, 4·78%; equiv. (by titration), 321; NH_2 (diazotizable), 5·05%; glucuronic acid, 54·2% (see above). $C_{13}H_{15}O_9N$ requires C, 49·8; H, 4·8; N, 4·47%; equiv., 313; NH_2, 5·12%; glucuronic acid, 62·1%.)

Further evidence that the compound first isolated was a complex of *p*-aminobenzoylglucuronide and *p*-aminobenzoic acid is provided by the fact that, when products were isolated which were syrups and could not be induced to crystallize directly, in most cases addition of *p*-aminobenzoic acid resulted in the crystallization of the complex.

o-*Aminobenzoylglucuronide.* The method described above gave a readily soluble syrup which could not be crystallized. It did not form a crystalline complex with anthranilic acid or with *p*-aminobenzoic acid nor has any crystalline derivative yet been obtained.

We were unable to isolate a glucuronide from *m*-aminobenzoic acid. Table 3 shows that this isomer is conjugated with glucuronic acid to a very much smaller extent than the others.

Isolation of oxidation products from o-*aminobenzamide*

This compound (30 g.) was administered to 20 rabbits and 24 hr. urine (8 l.) collected. This was hydrolyzed by boiling with conc. HCl (1/5 vol.) for 1 hr., made slightly acid to litmus and extracted continuously with ether for 48 hr., until the blue fluorescence was no longer visible in the ether layer. Two methods were attempted for the isolation of oxidation products from the ether-soluble material.

Chromatography. A solution of the ether-soluble material in ethanol and benzene was poured through an alumina column and anthranilic acid eluted with the same solvent mixture. Anthranilic acid could be readily distinguished from other metabolites in the column owing to its very strong fluorescence in ultraviolet light. It was, however, impossible to elute either with boiling water or organic solvents all the phenolic material which could be shown, by means of the $FeCl_3$ reaction, to remain on the column.

Fractional crystallization. The ether-soluble material was isolated by evaporation of the extract to dryness *in vacuo* and dissolved in 50% (v/v) aqueous ethanol. By progressive concentration under reduced pressure two main solid fractions were isolated and recrystallized from 60% (v/v) aqueous ethanol. The compounds isolated in all these experiments showed a great tendency to darken in air, especially in solution. The addition of a few drops of SO_2 water prevented this and enabled almost colourless products to be obtained.

The fractions obtained were as follows:

(1) Pale brown needles (yield 150 mg. from solid material extracted from 3 l. urine), soluble in ethanol only with difficulty. Purification was effected by continuous extraction with ether from an aqueous suspension. Ethereal solutions showed an intense blue-violet fluorescence in ordinary light. This compound darkened on heating and decomposed at 248°. It gave a brown coloration with $FeCl_3$, a positive indophenol reaction and reduced aqueous $AgNO_3$ solution in the cold. It gave no yellow coloration with HNO_2 and diazotized solutions did not couple normally with dimethyl-α-naphthylamine or with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, but in concentrated solutions (see p. 435) a violet colour, pale in the case of the first coupling agent and intense with the second, developed slowly, reaching maximum intensity in about 1 hr. With Br_2 water it gave a red-brown coloration. (Found: C, 54·8; H, 4·74; N, 9·2; calc. for $C_7H_7O_3N$; C, 54·8; H, 4·58; N, 9·14%.) The compound was identical in all respects with synthetic 5-hydroxyanthranilic acid prepared by reductive fission of 4-hydroxy-2-carboxyazobenzene in alkaline solution by means of $Na_2S_2O_4$ (cf. Limpricht, 1891). (Found: C, 54·6; H, 4·67; N, 9·09%; m.p. 248° (decomp.); Limpricht gives 235°; Bamberger & Pyman (1909) give 246–252° depending upon rate of heating.)

The method of preparation of the azo compound fixes only the relative position of the methyl and hydroxyl groups. The position of the amino group was established by conversion of the hydrochloride of the acid to *p*-aminophenol by sublimation *in vacuo*. This was characterized both as such and as its *N*-acetyl derivative. Both the synthetic and biosynthetic specimens of 5-hydroxyanthranilic acid were acetylated by treatment with acetic anhydride at ordinary temperature and in both cases crystalline products were obtained which melted, either alone or mixed together, at 238–239°. The compounds were shown to be monoacetyl derivatives inasmuch as they gave slight brown colorations with $FeCl_3$ and contained 6·62 and 6·95% N, respectively. Calc. for $C_9H_9O_4N$, 7·18%. Friedländer (1916) gives m.p. 227° and Kalle and Co. (1911), 224–226° (decomp.)

(2) This fraction on recrystallization yielded two compounds (*a*) and (*b*):

(*a*) Pale brown needles (yield 50–60 mg.), darkening at 230–240° and decomposing at 260°. This compound gave a dark brown coloration with $FeCl_3$, no indophenol reaction, no diazo reaction but an intense yellow colour with HNO_2. It was slightly reducing to aqueous $AgNO_3$ and gave no coloration with bromine water. In ethereal solution it showed a mauve fluorescence in ordinary light. The properties of this compound were identical with those of synthetic 3-hydroxyanthranilic acid. The only previous record of a compound to which this structure is attributed is due to Keller (1908), who prepared it by the action of red P and HI on damascenine, an alkaloid from *Nigella damascena*. The melting point is stated to be 163–164°, while our synthetic compound decomposed at 260°. Keller did not confirm the structure of his compound by synthesis, but the properties of our compound and its method of preparation suggest that it is, in fact, 3-hydroxyanthranilic acid. *m*-Acetamidobenzoic acid was nitrated as described by Kaiser (1885) to give 2-nitro-3-acetamidobenzoic acid, isolated in 20% yield as pale yellow needles, melting at 245° (Kaiser gives 240–241°). This compound was deacetylated and deaminated by boiling for about 1 hr. with aqueous KOH (25%) until the evolution of NH_3 ceased (cf. Griess, 1878), the 2-nitro-3-hydroxybenzoic acid formed being isolated in 70–80% yield, m.p. 162–163° (Griess found 178°). This compound was reduced with $SnCl_2$ and HCl by the method of Zahn (1900) and *3-hydroxyanthranilic acid* obtained as a slightly pink crystalline powder, darkening on heating to 240° and decomposing at 260°. It was a derivative of *o*-aminophenol inasmuch as its hydrochloride on sublimation *in vacuo* yielded this compound and since it gave no diazo reaction, but gave the typical yellow diazooxide colour with HNO_2. Further evidence of the configuration is provided by the fact that it, or its amide, is a metabolite of *o*-aminobenzamide. It contained 8·99% N; $C_7H_7O_3N$ requires 9·14%. The identity of the compound isolated from urine with this synthetic compound was confirmed by preparing an acetyl derivative by the action of acetic anhydride at ordinary temperature, and comparing it with the acetyl derivative of the synthetic compound, prepared in the same way. This compound does not appear to have been prepared before. Both the synthetic acetate and the acetate of the biosynthetic acid melted at 143–145°, either alone or mixed together. Both gave a slight dark brown coloration with $FeCl_3$, which suggests that the compound was the monoacetyl derivative. This was confirmed by the analysis of the synthetic specimen. It contained 7·14% N; $C_9H_9O_4N$ requires 7·18%.

(*b*) Pale brown needles (yield 20 mg.), decomposing at 230°. This compound was fairly soluble in aqueous ethanol, from which it was recrystallized, and gave no coloration with $FeCl_3$, negative indophenol and diazo reactions, a slight yellow coloration with HNO_2. Analysis gave C, 52·3; H, 4·62; N, 14·2%. The nature of this compound has not been determined. The analysis suggests that it might be an impure specimen of an aminohydroxyhippuric acid ($C_9H_{10}O_4N_2$ requires C, 51·4; H, 4·76; N, 13·34%), but it has not been possible to investigate it further.

The mother liquors from which these compounds were obtained were combined, evaporated to dryness *in vacuo* and chromatographed on alumina (see above). After elution of anthranilic acid, the presence of phenolic compounds on the column could be shown. Elution with boiling water enabled a further small amount of 5-hydroxyanthranilic acid to be isolated.

Isolation of oxidation products from m-*aminobenzamide*

To each of 19 rabbits 2 g. *m*-aminobenzamide were given, and the urine passed in the following 24 hr. was collected and hydrolyzed by boiling for 1 hr. with 1/5 vol. conc. HCl. The hydrolyzed urine was continuously extracted with ether for 48 hr., first at pH 6·5 and then at pH 5·5. The extracts obtained in this way had very similar properties and were recombined (extract *A*). The urine was then evaporated *in vacuo* to one-quarter bulk and again extracted with ether for 48 hr. (extract *B*). It was finally evaporated to dryness *in vacuo* at 60–70° and the residue extracted with 96% ethanol (extract *C*).

Extract *A* was evaporated to dryness, dissolved in water, treated with charcoal and progressively concentrated to yield various crystalline fractions and a syrupy residue (*D*). The crystalline fractions had indefinite melting points ranging from 165 to 195° and all gave positive diazo reactions and a brownish red colour with $FeCl_3$. They were

therefore recombined and recrystallized from 96% ethanol. Part of the material (500 mg.) did not dissolve easily. It contained some ash and was purified by extraction with ether from an acidified aqueous solution, followed by recrystallization from water. Yield 20 mg. of mauve needles, decomposing at 253°, which gave a purple coloration with $FeCl_3$, a negative diazo reaction, no coloration with HNO_2, a reddish brown coloration and precipitate with Br_2 water, a positive indophenol reaction, and reduced ammoniacal $AgNO_3$. These properties are those of 5-aminosalicylic acid. Recrystallization of the material which was soluble in 96% ethanol (above) yielded as a first fraction 30 mg. of white crystals whose properties, apart from colour, were the same as those of the compound just described. The identity of these two specimens with 5-aminosalicylic acid was confirmed by conversion into the *NO*-diacetyl derivative, m.p. 192°, which did not depress the melting point (194°) of an authentic specimen of 5-acetamidoacetylsalicylic acid. Meldola, Foster & Brightman (1917) give m.p. 283° for the acid and 197° for the acetate (actually the diacetate; present authors unpublished). The remainder of the ethanol-soluble material (6 g.) consisted of *m*-aminobenzoic acid. The syrupy residue *D* was treated with acetic anhydride, dissolved in water and fractionated. Three fractions were obtained: (1) *m*-acetamidobenzoic acid (1 g.); (2) a crystalline fraction of lower melting point (1 g.) which on deacetylation and fractionation with ethanol yielded mainly *m*-aminobenzoic acid; (3) a syrupy residue (2 g.) which was chromatographed on alumina, using ethanol-benzene as solvent. The fractions thus obtained after deacetylation gave a purple-brown coloration with $FeCl_3$ and a slight yellow colour with HNO_2, but contained relatively large amounts of *m*-aminobenzoic acid which prevented the isolation of the phenolic compound present.

Extract *B*, treated in the same way as extract *A*, yielded three main fractions: (1) white crystals (40 mg.) giving a purple coloration with $FeCl_3$, a positive diazo reaction, developing slowly, no yellow colour with HNO_2 and a bright-green colour and precipitate with Br_2 water. Analysis gave: C, 49·2; H, 5·1; N, 17·7%. This compound was neither an acid nor an ammonium salt. It did not melt, but partially sublimed with decomposition at 310°. Its identity has not yet been established, but it is suggested that it might be a hydrated amide of an aminohydroxybenzoic acid. $C_7H_6N_2O_3.H_2O$ requires C, 49·4; H, 5·9; N, 16·5%; (2) white needles (200 mg.) which did not melt below 300° and contained an appreciable amount of ash; 150 mg. were acetylated and gave a compound which was proved to be 5-acetamidosalicylic acid. Sublimation *in vacuo* of the remainder yielded crystalline *p*-aminophenol, presumably derived from 5-aminosalicylic acid by decarboxylation; (3) a syrup, which on chromatography (as above) yielded 10 mg. crystals and a syrupy fraction. After recrystallization from water the crystals melted at 215° (decomp.) and gave a yellow colour with HNO_2, but a negative diazo reaction. With $FeCl_3$ they gave a bluish purple coloration. It is probable that this compound was 3-amino-4-hydroxybenzoic acid, but, owing to the small amount isolated, it was not possible to characterize it finally. The syrup gave a purple-brown coloration with $FeCl_3$, but contained also an appreciable amount of *m*-aminobenzoic acid which prevented isolation of the substance responsible.

Extract *C* gave a purple colour with $FeCl_3$, but no metabolite of *m*-aminobenzamide could be isolated.

Isolation of oxidation products from p-*aminobenzamide*

p-Aminobenzamide (30 g.) was fed to 12 rabbits and 24 hr. urine collected. This was hydrolyzed, neutralized and extracted as in the case of *o*-aminobenzamide. The ether-soluble material was recrystallized from aqueous ethanol and a considerable amount of *p*-aminobenzoic acid recovered. The mother liquors gave a bluish coloration with $FeCl_3$ and ultimately yielded about 15 mg. of a white crystalline material which gave a dark-blue coloration with $FeCl_3$, a negative diazo reaction and an intense yellow coloration with HNO_2. It decomposed at 225–227°. These properties are identical with those of synthetic 4-amino-3-hydroxybenzoic acid, prepared by the nitration of *m*-hydroxybenzoic acid (Griess, 1887) and reduction of the resulting 4-nitro-3-hydroxybenzoic acid with $SnCl_2$ and HCl (cf. Zahn, 1900). M.p. 226° (Einhorn & Pfyl (1900) give 216°). The identity of the synthetic and biosynthetic acids was confirmed by comparing their *acetyl* derivatives. That of the compound from urine melted (with decomposition) at 238–239°, that of the synthetic compound at 242–244° and a mixture of the two at 240–241°. This acetate does not appear to have been prepared previously.

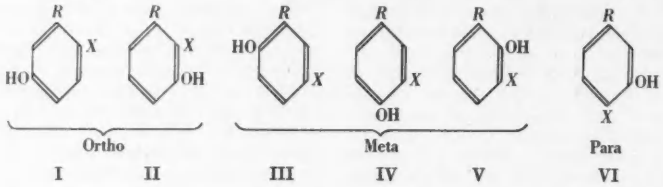
It should be noted that although these oxidation products were isolated and characterized as acids, it is probable that they are excreted as amides (see p. 434). It is reasonable to conclude that the introduction of the hydroxyl group takes place while the carbamyl group is intact, since the acids themselves give rise to no increased excretion of ethereal sulphate. Whether or not hydrolysis of the carbamyl group takes place more readily in aminohydroxybenzamides than in aminobenzamides is not yet known.

DISCUSSION

The results given in Tables 1–4 and summarized in Table 5 show clearly that there is a considerable difference between the metabolism of the aminobenzoic acids and their amides. Differences in oxidation (as shown by the increase in ethereal sulphate excretion) and in acetylation provide indirect evidence of the comparative stability of the amide grouping in these compounds *in vivo*, while the isolation of unchanged *o*-aminobenzamide, and of *N*-acetyl derivatives of the *m*- and *p*-isomers from the urine of rabbits which had received the amides indicates directly that hydrolysis of the carbamyl group is not complete. Estimations of ether-soluble acid indicate that the group is hydrolyzed to an extent of about 12–33% in the *o*-, 8–12% in the *m*- and 15–21% in the *p*-amide. The corresponding percentages of hydrolysis obtained *in vitro* with liver extracts were 9, 7 and 13 (Bray *et al.* 1948).

The identity of the oxidation products isolated is of particular interest inasmuch as the generalization suggested in a previous paper (Bray *et al.* 1947) can now be more clearly defined. Taking into account the work of other investigators and work in this laboratory, it appears to be a general rule that, in

the hydroxylation *in vivo* of a disubstituted aromatic compound, $R.C_6H_4.X$, where R is COOH, $CONH_2$, SO_2NH_2 or CH_3 and X is OH or NH_2 ($NHCOCH_3$ in acetotoluidides), the position of the hydroxyl group introduced is, as might be expected on purely chemical grounds, either para or ortho to X, giving, when R and X are themselves ortho, I or II; when R and X are meta, III or IV (V has not yet been reported) and, when R and X are para, VI.



It has been proved in the present investigation that in the case of *o*-aminobenzamide the two possible oxidation products, I and II, are both formed. Evidence for the formation of the two expected oxidation products of orthanilamide has been put forward by Stubbs & Williams (1947). From *m*-aminobenzamide the main oxidation product isolated corresponded to III. There was, however, evidence of another oxidation product which was probably IV. From *p*-aminobenzamide the only oxidation product isolated was, as would be expected, VI.

The case of the acetotoluidides is of interest since we have shown (unpublished result) that in the rabbit the *o*-isomer gives rise mainly to 2-amino-5-hydroxytoluene (6-amino-*m*-cresol) (I), excreted both as glucuronide and ethereal sulphate, whereas Jaffe & Hilbert (1888) found that the dog excreted a benzoxazolone derivative, presumably derived from 2-amino-3-hydroxytoluene (2-amino-*m*-cresol) (II). We cannot yet say whether this isomer is also formed by the rabbit. In the *m*- and *p*-acetotoluidides we have confirmed Jaffe & Hilbert's (1888) observation that the oxidation of the methyl group takes precedence, aminobenzoic acids appearing as the main excretion products. Phenolic compounds are, however, formed in smaller amounts, particularly with the *m*-isomer, but have not yet been identified. All these compounds are actually excreted as *N*-acetyl derivatives, since only a small amount of deacetylation takes place *in vivo*.

It would be expected that in the compounds under discussion the group X (OH or NH_2) will determine the position of the entering hydroxyl group, group R having no significant directing effect. The hydroxylated compounds, in fact, correspond to those formed when the group R is a hydrogen atom, i.e. in monosubstituted compounds. Phenol is converted in the animal to catechol and hydroquinone (Baumann & Preusse, 1879) and aniline (as acetanilide or formanilide) to *o*-aminophenol (as benzoxazolone) in the dog and to *p*-aminophenol in the rabbit (Jaffe & Hilbert, 1888; Kleine, 1896–7). Similar results have been observed if group X is modified, e.g. phenetole is converted to monoethyl hydroquinone in the dog (Kossel, 1880); β-phenylhydroxylamine and cupferron (ammonium nitrosophenylhydroxylamine) to *p*-aminophenol in the rabbit (Sieburg, 1914) and dimethylaniline to *o*-aminophenol in the dog and to *p*-monomethylaminophenol in the rabbit (Horn, 1936*a*, *b*). We have also shown (unpublished result) that phenylurea is converted in the rabbit to *p*-hydroxyphenylurea, which is excreted mainly as glucuronide. So far as we are aware, compounds in which the group X and a hydroxyl group occupy positions meta to one another have never been found as products of mammalian metabolism.

SUMMARY

1. The metabolism of *o*-, *m*- and *p*-aminobenzoic acids and their amides in the rabbit has been studied both qualitatively and quantitatively.

2. There is evidence that the carbamyl group is hydrolyzed in the rabbit to an extent of 12–33% in the *o*-, 8–12% in the *m*-, and 15–21% in the *p*-compound.

3. Among the compounds isolated were *m*-acetamidobenzamide and *p*-acetamidobenzamide as metabolites of the amides themselves and *p*-aminobenzoylglucuronide as a metabolite of *p*-aminobenzoic acid. This glucuronide forms a crystalline complex with *p*-aminobenzoic acid.

4. Four aminohydroxybenzoic acids have been isolated from the hydrolyzed urines of rabbits which had received the aminobenzamides under investigation. In this way 3- and 5-hydroxyanthranilic acids were obtained from *o*-aminobenzamide, 3-amino-6-hydroxybenzoic acid from *m*-aminobenzamide and 4-amino-3-hydroxybenzoic acid from *p*-aminobenzamide.

5. This type of biological hydroxylation is discussed from the point of view of the position taken up by the hydroxyl group introduced with respect to the other groups present.

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Separation of Acidic Amino-acids by means of a Synthetic Anion Exchange Resin

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Synthetic ion-exchange resins have recently been widely used for the separation of basic and acidic from neutral compounds. Thus Cannan (1944), by repeated applications of Amberlite IR4, separated glutamic and aspartic acids quantitatively from protein hydrolysates. Earlier, Freudenberg, Walch & Molter (1942) had used a column of Wolfatite M for the separation of the dicarboxylic amino-acids, and Cleaver, Hardy & Cassidy (1945) investigated the chromatographic separation of amino-acids by organic exchange resins. Recently, Tiselius, Drake & Hagdahl (1947) have employed synthetic ion exchange resins chromatographically for the quantitative separation of amino-acids into groups. The aim of the present investigation was to extend such methods to cover the separation of the acidic amino-acids from one another (at pH 2·5) after their isolation from the rest of the protein hydrolysate (at pH 3–4). Although completely quantitative recoveries were not obtained, complete separations have been achieved. The separation depends on differences in net charge between the acidic amino-acids at a given pH (Fig. 1). Thus, glutamic acid with pK_1 2·16 and pK_2 4·32 (Neuberger, 1936) has a net charge of $-0{\cdot}31$ at pH 4; aspartic acid with pK_1 1·88 and pK_2 3·65 (Miyamoto & Schmidt, 1931) has a net charge of $-0{\cdot}69$ at pH 4; whilst cysteic acid with pK_1 1·88 and pK_2 1·30 (Andrews & Schmidt, 1927)

has a net charge of −0·99 at this pH. It would therefore be expected that a column of Amberlite IR4 maintained uniformly at pH 4, because of its positive charge, will preferentially retain cysteic, aspartic and glutamic acids in this order. Theoretically, it would appear that the optimum pH for the separation of glutamic from aspartic acid is about 4, since at this pH the largest difference in net charge occurs, but experimentally it was found that for satisfactory separation a pH of 2·5 was necessary. At this pH glutamic and aspartic acids normally carry a net positive charge of +0·29 and +0·13 respectively. Nevertheless, at this pH they are retained on Amberlite IR4 columns and can be separated by elution in relatively small volumes; at higher pH values, although separation will take place, relatively large volumes are required. This suggests that the pK values are depressed when these amino-acids are adsorbed on Amberlite IR4. Similarly, cysteic acid is partially retained at pH 1·5, although its net charge at this pH should be almost zero.

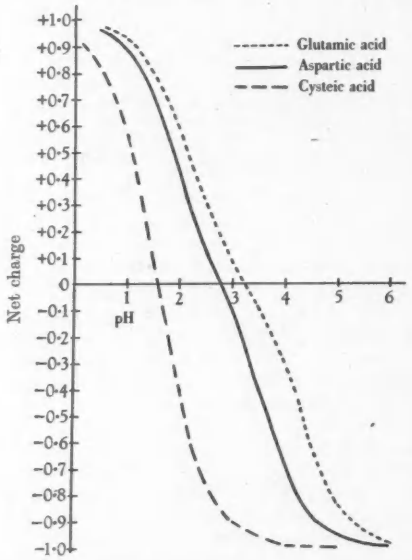

Fig. 1. Net charge of glutamic, aspartic and cysteic acids at different pH values, calculated from the dissociation constants given in the text.

To apply these principles, care has been taken to bring the Amberlite to the required pH and to maintain it uniformly in that part of the column where separations are taking place. In order to establish equilibrium conditions, the resin was finely powdered and the width of the column kept small in relation to its height so that low rates of flow were maintained. In the first stage, no difficulty was found in effecting the separation of the acidic from the basic and neutral amino-acids in a protein hydrolysate at pH 3–4. As is shown by its titration curve (Fig. 2), at this rather acid pH, the percentage charge on the resin is sufficiently high for it to compete successfully with lysine and arginine for the acidic amino-acids. The departure from the expected sigmoid shape of the curve below pH 1 may be due to decomposition of the resin. This is also suggested by the fact that the nitrogen (Kjeldahl) washed from a column by acid is about ten times as high for strong acid (pH 0–1) as for weak acid (pH 3–4) and is independent of the history (previous washings) of the column. By the above means and by the application of the chromatographic principle, considerable economy of resin was achieved. Separations have been followed by partition chromatography on paper (Consden, Gordon & Martin, 1944) of samples from each eluate; this method has provided a convenient means for identifying the amino-acids present in each fraction, without using more than negligible quantities.

The copper titration method of Pope & Stevens (1939) was chosen as a convenient means of estimating the acidic amino-acids after separation by the resin and was adapted to deal with micro quantities. Unfortunately, it was not found possible to obtain fully quantitative recoveries of glutamic and aspartic acids after their separation from synthetic mixtures. The recovery of glutamic acid was about 91 %, that for aspartic about 96 %. A possible cause for low recoveries may be formaldehyde which appears to be continually liberated even after the washing cycle employed by Cannan (1944). Attempted removal of formaldehyde failed to improve the results. Estimation of amino N or total N in the eluates by the Van Slyke manometric procedure or Kjeldahl method was tried and abandoned owing to the high blanks given by eluates washed off the column after the removal of amino-acids. Cysteic acid, as isolated from hydrolysates by means of Amberlite, is always accompanied by pigment having a similar acid strength. As similar pigment material from hydrolysates of wool not containing cysteic acid gives significant titrations in the Pope & Stevens (1939) procedure, estimations of cysteic acid would presumably give fictitiously high results.

As the synthetic ion exchange resins can absorb relatively large amounts of amino-acids (Englis & Fiess, 1944; Cleaver *et al.* 1945), they are suitable for isolations. Thus, cysteic acid has been isolated from a hydrolysate of chlorinated wool. This material was almost fully optically active, whereas that obtained by Lissizin (1928) from hair oxidized by alkaline permanganate was inactive.

METHODS

*Preparation of the Amberlite IR4.** The resin is alternately ground wet in a ball mill and sieved with a jet of water, −90 +200 grading being used for the columns. It is washed free from NH_3, treated with HCl until the supernatant liquor is permanently acid to thymol blue (pH <1) and then washed with water. Alternate treatments with acid and water are repeated a number of times.

Preparation of columns. (i) The resin is stirred and washed by decantation with water until the supernatant liquid is less than 0·001 N with respect to alkali. The slurry is transferred to a glass tube of 0·5 cm. internal diameter, forming a column 20 cm. in height (containing about 2 g. air-dry resin), and washed overnight with water at a rate of approx. 15 ml./hr. This does not significantly raise the pH of the eluate, the acid concentration of which should be checked by titration before the next stage. (ii) The second column (height 30 cm.) is prepared from about 3 g. Amberlite and the pH is adjusted accurately to 2·5 by equilibration with dilute acid or water until the acidity of the supernatant fluid is slightly above 0·003 N. The material is then transferred to a 0·5 cm. diameter tube and washed overnight with 0·003 N-HCl. The acidity of the eluate is finally checked by titration. The columns should not be allowed to run dry and remain so for any length of time; otherwise extensive cracking may occur.

Procedure for estimation of glutamic and aspartic acids. The hydrolysate prepared with HCl and containing up to 15 mg. acidic amino-acids is repeatedly evaporated *in vacuo* by rotating manually in a warm water bath, allowed to stand overnight *in vacuo* over KOH, and quantitatively transferred in a small volume to the first column. Water is then passed down the column; the neutral and basic amino-acids are eluted in the first 25 ml. The next 5 ml. should be free from amino-acids. To test the eluates, a portion of the first 25 ml. and the whole of the second 5 ml. fraction are separately concentrated *in vacuo* to very small bulk and samples of each tested by partition chromatography on a strip of Whatman no. 4 paper using phenol-NH_3, developing for 5 hr. or more, with suitable control (cf. Consden *et al.* 1944). After development and treatment with ninhydrin, basic and neutral, but no acidic amino-acids, are found in the first eluate, whilst the second eluate should contain no amino-acids. If cystine is present, the eluate sample is oxidized by treatment with excess Br_2 water before chromatography; the cysteic acid thus produced occupies a position on the chromatogram not overlapping the positions of aspartic and glutamic acids (Consden, Gordon & Martin, 1946) if these are present.

The next stage consists in transferring the material remaining in the column to the second column. This may be done in either of two ways:

Method 1. The column is eluted with N-HCl causing the pigment to run down as a sharp band. When this has run out, the washing is stopped and the eluate repeatedly evaporated *in vacuo* and allowed to stand overnight *in vacuo* over KOH to remove excess HCl. It is then quantitatively transferred to the second column which is eluted with 0·003 N-HCl, 10 ml. eluates being collected. Each is concentrated *in vacuo* to a volume of 1–2 ml. and a sample (4 μl.) tested as described above.

* Obtained from the Resinous Products and Chemical Co., Pa.

Method 2. The column is washed with 3 ml. N-HCl, causing the pigment band to travel about half way down. (The eluate should be free from amino-acids as revealed by chromatography.) The column is then fixed to the top of the second column. Elution is continued with water, but the acid already added is sufficient to cause the pigment band to continue to travel down and eventually to pass into the lower column. When the pigment has travelled a few cm. into the second column, the top column is removed and elution of the lower column continued with 0·003 N-HCl. If no pigment is present, elution may be followed by the change in shade of the resin which is orange when strongly acid. Eluates are collected and tested as in method 1.

Method 1 has been found to behave more consistently than method 2; in proteins where the ratio of glutamic to aspartic acid is of the order 2 : 1, the glutamic acid is eluted from the second column in the first 20 ml., and aspartic acid in the next 30 ml., whereas in method 2 the stages at which glutamic and then aspartic acids begin to appear are rather less certain. Method 2 is quicker than method 1 but requires more attention.

Each eluate is evaporated as above *in vacuo* to dryness, and a known volume (5–10 ml.) of copper phosphate-buffer suspension (Pope & Stevens, 1939) is added to the crystalline residue. Titrations (20–50 μl. 0·1 N-$Na_2S_2O_3$) are carried out on 1 ml. portions after filtering and addition of the other reagents. Neutralization before addition of the copper phosphate is unnecessary.

Procedure for the isolation of cysteic acid. In order to assess the amount of protein from which a workable quantity of cysteic acid may be isolated, its amount in a hydrolysate is roughly estimated by partition chromatography on paper. A known volume (5–10 μl.) is applied to Whatman no. 4 paper and known amounts (0·5–20 μg.) of cysteic acid, each at the same volume as that of the hydrolysate, applied by the side of the hydrolysate spot. After development of the chromatogram in phenol-NH_3-coal gas for 17 hr., the paper is dried, coloured with ninhydrin and the depth of colour of the cysteic acid spot in the hydrolysate compared with those of the controls. The isolation may be conducted either (*a*) by eluting the column with N-HCl after separation of the dicarboxylic amino-acids by method 1 or 2, or (*b*) at pH 1·5. If (*a*) has been used, partition chromatography reveals that the whole of the cysteic acid runs out in the region of the pigment band, at which stage there is a sharp change in pH from 2·5 to <1. No complete separation of the pigment from cysteic acid was achieved by using the resin and eluting solution at a lower pH; however, at pH 1·5 all the amino-acids except cysteic acid are rapidly eluted, thus making possible its isolation on only one column. Thus, on a column, length 30 cm., diameter 0·5 cm., eluted with 0·03 N-HCl, the neutral, basic and dicarboxylic amino-acids accompanied by some pigment, run out in the first 25 ml. followed soon after by cysteic acid, the elution of which can be speeded up by N-HCl.

RESULTS

Recovery of glutamic and aspartic acids from a mixture of amino-acids

An amino-acid mixture simulating a wool hydrolysate (Astbury, 1942) was prepared containing 0·0517 mg. aspartic acid N (4·12 % of total N) and

Table 1. *Recoveries of glutamic and aspartic acids from a synthetic mixture*

	Amount (mg. N)	Experiment (i) Found	(i) Recovery (%)	(ii) Found	(ii) Recovery (%)	(iii) Found	(iii) Recovery (%)	(iv) Found	(iv) Recovery (%)
Glutamic acid	0·8416	0·767	91·3	0·773	92·0	0·776	92·3	0·747	88·8
Aspartic acid	0·4126	0·394	95·2	0·390	94·2	0·404	97·6	0·397	96·0

0·1052 mg. glutamic acid N (8·40 % of total N)/ml. This solution (8 ml.), after evaporation *in vacuo*, was subjected to the foregoing procedure, using method 2. Recoveries of glutamic and aspartic acids are recorded in Table 1.

In experiments on glutamic and aspartic acids alone, similar recoveries were obtained. The odour of formaldehyde could usually be detected in the eluates from the columns after concentration; treatment of the eluates with alkali, HCl, H_2O_2, or dimedone failed to improve the results and treatment with strong NH_3 gave high results. Addition of formaldehyde to glutamic and aspartic acid solutions led to low titrations.

Isolation of cysteic acid

Degraded protein produced by the action of chlorine on wool (Consden *et al.* 1946), containing 156 mg. N and which by the paper chromatographic method described above was expected to contain approx. 4 mg. cysteic acid N, was hydrolyzed and prepared as already described for application to the resin. This material was transferred to a column (30 cm. high; 1·5 cm. diam.) which had been equilibrated to pH 1·5 and was then eluted with 0·03 N-HCl, the rate of flow through the column being about 60 ml./hr. Six eluates of 20 ml. each were collected and tested as described above. The neutral amino-acids plus glutamic and aspartic acids were eluted in the first three fractions. The fourth fraction contained much cysteic acid and subsequent fractions successively less. The column was eluted with a further 500 ml. 0·03 N-HCl and, as small amounts of cysteic acid were still being washed out, it was finally eluted with N-HCl which removed all the remaining cysteic acid and yellow material from the resin. The united fractions containing cysteic acid were concentrated *in vacuo* to small volume and the solution, after standing at 0°, deposited brownish crystals, which after filtration were decolorized by boiling with animal charcoal. The mixture was filtered, evaporated *in vacuo* to dryness and recrystallized three times from aqueous ethanol. The pale yellow powder (15 mg.) had $[\alpha]_D^{18°} + 7·1°$ (*l.* 2; $c = 0·3$) in water. Clarke & Inouye (1931) give $[\alpha]_D^{24·5°} + 9·4°$ for L-cysteic acid. Its equivalent by titration with $Ba(OH)_2$ was 185; calc. for $C_3H_7O_5N.H_2O$, 187; mixed partition chromatograms using phenol-NH_3-coal gas showed no difference from authentic L-cysteic acid. Analysis of the copper salt gave Cu 25·78; calc. for $C_3H_7O_6NSCu$, Cu, 25·57 %.

Titration of Amberlite IR 4

The resin, which had been treated as described at the beginning of the experimental section, was brought to *c.* pH 10 by treatment with dilute NaOH. It was then thoroughly washed with water and left to dry at room temperature. A sample of the resin (202·8 mg.) on drying to constant weight at 105° lost 30·4 mg. Samples of air-dry resin (200 mg.) were mixed with varying quantities of HCl and each made up to a final volume of 20 ml. and allowed to equilibrate overnight. The pH of the filtrate was determined (glass electrode) and acidity by titration against alkali. The absorption of acid by Amberlite IR 4 at different pH values is shown in Fig. 2.

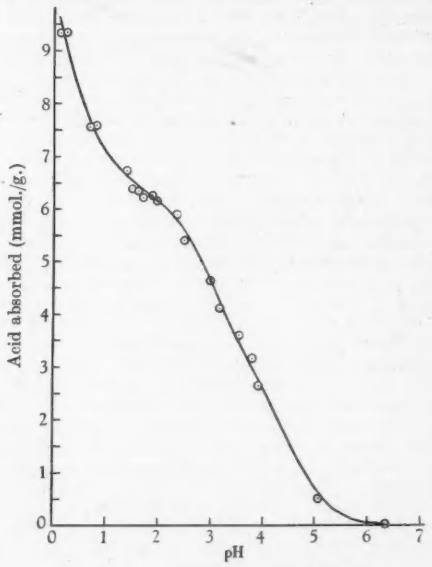

Fig. 2. Titration curve of Amberlite IR 4.

SUMMARY

1. A simple chromatographic method is presented for the separation of glutamic from aspartic acid, and their estimation, after their separation together from a protein hydrolysate, employing the synthetic anion exchange resin, Amberlite IR 4.

2. Optically active cysteic acid was isolated from a hydrolysate of chlorinated wool.

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The Polarographic Estimation of Steroid Hormones

4. DETERMINATION OF 3(α)- AND 3(β)-HYDROXY-17-KETOSTEROIDS

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A knowledge of the urinary excretion of 3(β)-hydroxy-17-ketosteroids ('β-17-ketosteroids') is becoming increasingly important in clinical diagnosis. In addition to the now well-established fact, first suggested by Crooke & Callow (1939) and by Talbot, Butler & Berman (1942), that the relative excretion of β-17-ketosteroids is markedly elevated in patients with adrenal cortical carcinoma, the work of Scott & Vermeulen (1942), Pearlman (1942), Dobriner, Gordon, Rhoads, Lieberman & Fieser (1942), Freeman, Pincus & Glover (1944), Salter, Cahen & Sappington (1946) and Dingemanse, Huis in't Veld & de Laat (1946) has shown that the excretion of β-17-ketosteroids may be altered in various other cancerous and non-cancerous conditions. It therefore appeared desirable to extend the polarographic method for the estimation of 17-ketosteroids (Barnett, Henly & Morris, 1946) so that it could also be used for the routine estimation of the α- and β-17-ketosteroids.

Several methods have been described for the quantitative separation of the 3(α)- and 3(β)-hydroxy fractions of urinary 17-ketosteroid extracts. All depend upon the use of digitonin for precipitation of the β-fraction. The procedure of Talbot, Butler & MacLachlan (1940*a*) is not entirely satisfactory in so far as it relies on an indirect determination of the β-fraction. The micro-method of Baumann & Metzger (1940) has been criticized by Salter *et al.* (1946) on the grounds that several conditions (e.g. ethanolic content of the medium, and initial concentration of steroid) may alter the apparent partition between α- and β-fractions. The procedure of Frame (1944) has the single disadvantage that about 15 mg. of total steroid are required, an amount which would necessitate the extraction of several days' collection of urine from subjects with a low 17-ketosteroid excretion. Pincus (1945) described a micro-procedure (using about 1 mg. of total steroid) and suggested the addition of dehydro*iso*androsterone (3(β)-hydroxyandrost-5-en-17-one) in order to obtain maximum precipitation of the β-fraction. He also commented that, regardless of the β-17-ketosteroid content, digitonin precipitation leads to a loss of approximately 0·02 mg. of β-17-ketosteroid to the α-fraction. There is evidently a need for a method of separation of α- and β-17-ketosteroids in urine extracts, which would combine the reliability of

Frame's procedure with the convenience of a micro-method. The method described below (based mainly on the methods of Frame, 1944 and of Pincus, 1945) has been developed with these objects in mind. It has been used in the determination of the excretion of α- and β-17-ketosteroids in a series of ten normal men and ten normal women.

EXPERIMENTAL

Collection and treatment of urine

Ten men (medical students and laboratory staff) and ten women (nurses) provided 24 hr. collections of urine. The specimens were collected in vessels containing 7 ml. of conc. HCl, and the importance of accurate collection was stressed. The specimens were kept in a cool place during collection and then stored in a refrigerator until extracted, generally within 24 hr.

The urines were hydrolyzed and extracted according to the method described by Barnett, Henly & Morris (1946), and the extracts were separated into α- and β-fractions by the procedure described below. The final extracts were oxidized by permanganate-periodate, treated with Girard's reagent *T* and polarographed as described by Barnett, Henly & Morris (1946). The dropping electrode used in the course of this work had the following constants: drop time *t*, 1·68 sec.; average drop weight, 5·35 mg.: capillary constant $k = m^{\frac{2}{3}} t^{\frac{1}{6}} = 2{\cdot}36$ (m = mass of mercury dropping/sec.). Androsterone and 3(β)-hydroxyandrost-5-en-17-one were found to give polarograms with identical wave heights (100 μg. of each gave a wave height of 17·5 mm. at sensitivity C (cf. Barnett, Henly & Morris, 1946)).

Separation of urinary 17-ketosteroids into α- and β-fractions

Reagents. Digitonin solution. A 1% (w/v) solution of digitonin (British Drug Houses, Ltd.) in warm 90% (v/v) ethanol. The digitonin crystallizes from the solution on standing at room temperature, but it may be kept in solution by storing at 37° in a tightly stoppered container. *Ether*: The ether must be peroxide free. Howard's Standard Ether (*Aether Anaestheticus P.B.*) has been found satisfactory. *Pyridine*: The 'Analar' reagent is dried over barium oxide and redistilled at frequent intervals. *Sulphuric acid*: Approx. 2N.

Procedure. A portion of benzene solution of urine extract, containing 1·0–1·5 mg. of 17-ketosteroid, is transferred to a graduated centrifuge tube and evaporated to dryness, preferably under reduced pressure. Warm digitonin solution (0·75 ml.) is added and the mixture quickly heated to boiling (over a microflame) in order to dissolve as much of the material as possible. The tube is stoppered and left in a refrigerator overnight. A total of 10 ml. of peroxide-free ether is then added in small portions, stirring with a thin glass rod between each addition, and allowing the precipitated digitonin and digitonides to flocculate before adding the last 2–3 ml. The mixture is centrifuged for 5 min. at 2000–3000 r.p.m. and the supernatant liquid decanted into a separating funnel of 30–50 ml. capacity. The precipitate in the centrifuge tube is washed three times with 5 ml. portions of ether, stirring and centrifuging each time, as before. The combined supernatant liquids are washed three times with 5 ml. portions of water, transferred to a small flask, the ether removed under reduced pressure, and the residue dried *in vacuo* over $CaCl_2$ for about 1 hr. This constitutes the α-fraction.

The residue in the centrifuge tube is dissolved in 0·25 ml. of dry pyridine and warmed in a water bath at 60–70° for 3 min. After cooling in running water, 5 ml. of ether are added in small quantities, stirring between additions and allowing the precipitate to flocculate as before. The mixture is centrifuged for 5 min. and the supernatant liquid poured off into a small separating funnel. The residue in the centrifuge tube is again treated with pyridine and ether, and the final precipitate washed twice with 5 ml. portions of ether. The combined pyridine-ether extracts are washed twice with 5 ml. portions of 2N-H_2SO_4 and three times with 5 ml. portions of water. After transferring to a small flask, the ether is removed under reduced pressure and the residue dried *in vacuo* over $CaCl_2$ for about an hour. This constitutes the β-fraction.

With normal urines it will usually be necessary to use about one-tenth of the α-fraction and the whole of the β-fraction for oxidation and polarography. In both cases optimal results are obtained when about 60–120 μg. of 17-ketosteroids are present.

A few experiments have been carried out in which β-fractions obtained from the same urine extract have been polarographed with and without previous oxidation. The results indicate that little improvement may be obtained by oxidation, and further work may show that oxidation of β-fractions is, in fact, unnecessary. It would appear that most of the interfering material removed by oxidation remains with the α-fraction.

RESULTS

Tables 1 and 2 show the results of experiments to test the accuracy of the procedure. In each case the ethanolic solution of pure sterol or urine extract or mixture was evaporated to dryness in a graduated centrifuge tube and separated into α- and β-fractions. Androsterone was used as the α-17-ketosteroid and 3(β)-hydroxyandrost-5-en-17-one as the β-17-ketosteroid. It will be seen that in all cases the recoveries of both fractions are satisfactory.

Table 1. *Recovery of α- and β-17-ketosteroids from pure solutions*

	α-17-Ketosteroids			β-17-Ketosteroids		
		Recovered			Recovered	
Exp. no.	Added (μg.)	(μg.)	(%)	Added (μg.)	(μg.)	(%)
1	—	—	—	100	95	95
2	—	—	—	100	100	100
3	—	0	—	200	200	100
4	100	97	97	100	100	100
5	100	100	100	100	100	100
6	100	97	97	200	194	97
7	60	65	108	100	97	97
8	75	71	95	60	61	102
9	200	200	100	88	80	100
10	400	377	94	50	47	94
	Means		98·7			98·5

Table 2. *Recovery of α- and β-17-ketosteroids from urine extracts*

	α-17-Ketosteroids				β-17-Ketosteroids			
			Recovery of added material				Recovery of added material	
Exp. no.	Present (μg.)	Added (μg.)	(μg.)	(%)	Present (μg.)	Added (μg.)	(μg.)	(%)
11	914	400	401	100	29	100	91	91
12	914	500	516	103	29	50	50	100
13	914	600	571	95	29	150	141	94
14	1255	150	145	97	36	200	193	97
15	1255	0	0	—	36	500	464	93
16	857	279	283	102	57	300	303	101
17	1614	335	356	106	140	400	414	105
			Means	100·5				97·9

Table 3. *The separation of the 17-ketosteroids in a pooled urine extract into α- and β-fractions, at different concentration levels*

Vol. of extract used (ml.)	Total 17-ketosteroids (μg./ml.)	α-17-Keto-steroids (μg./ml.)	β-17-Keto-steroids (μg./ml.)	(α + β)-17-Keto-steroids (μg./ml.)	(α + β)/total 17-ketosteroids (%)
0·8	1400	1360	82	1442	103·0
1·5	1400	1335	84	1419	101·4
2·5	1400	1300	89	1389	99·2

Table 4. *The urinary excretion of total, α- and β-17-ketosteroids in ten normal men*

Extract no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (% of total)	Recovery (α + β)/total 17-ketosteroids (%)
MS 1	20	2260	15·1	12·9	0·8	5·3	91
MS 2	20	2270	13·4	10·6	2·0	14·9	94
MS 3	27	1790	21·8	19·3	1·7	7·8	96
MS 4	24	1850	16·9	15·6	0·6	3·6	96
MS 5	31	1920	10·4	9·6	0·8	7·7	100
MS 6	25	950	19·7	18·3	1·2	6·1	99
MS 7	38	1670	11·9	10·4	0·9	7·6	95
MS 8	32	2600	16·3	14·7	0·3	1·8	92
MS 9	31	1310	11·2	9·4	0·7	6·3	90
MS 10	25	1040	15·9	14·7	0·4	2·5	94
Means			15·3	13·6	0·9	6·4	95
Standard deviations (σ)			±3·7	±3·6	±0·5	±3·7	±3
Probable range (mean ±2σ)			7·9–22·7	6·4–20·8	0–1·9	0–13·8	89–101
Standard deviation among duplicates				±0·5	±0·03		

Table 5. *The urinary excretion of total, α- and β-17-ketosteroids in ten normal women*

Exp. no.	Age (yr.)	Volume of urine (ml./24 hr.)	Total 17-ketosteroids (mg./24 hr.)	α-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (mg./24 hr.)	β-17-Keto-steroids (% of total)	Recovery (α + β)/total 17-ketosteroids (%)
WS 1	22	1700	21·4	19·4	1·6	7·5	96
WS 2	20	1200	14·3	13·6	0·6	4·2	99
WS 3	21	1680	10·1	9·4	0·8	7·9	101
WS 4	20	820	8·5	7·7	0·4	4·7	95
WS 5	22	1350	8·5	8·3	0·2	2·4	100
WS 6	18·5	1860	11·9	10·9	0·4	3·4	95
WS 7	21	1250	15·7	15·9	0·4	2·5	104
WS 8	21	775	14·5	13·1	1·0	6·9	97
WS 9	24	710	15·1	14·1	1·0	6·6	100
WS 10	19	2500	20·7	20·2	0·5	2·4	100
Means	20·9	—	14·1	13·3	0·7	4·9	98
Standard deviations (σ)			±4·5	±4·3	±0·4	±2·1	±3
Probable range (mean ±2σ)			5·1–23·1	4·7–21·9	0–1·5	0·7–9·1	92–104
Standard deviation among duplicates				±0·6	±0·03		

Table 6. *The normal urinary excretion of α- and β-17-ketosteroids as reported by various workers since* 1940

Authors	No. of cases	Sex	α-17-Ketosteroids (mg./24 hr.) Mean	α-17-Ketosteroids (mg./24 hr.) Standard deviation (σ)	α-17-Ketosteroids (mg./24 hr.) Range (mean $\pm 2\sigma$)	β-17-Ketosteroids (mg./24 hr.) Mean	β-17-Ketosteroids (mg./24 hr.) Standard deviation (σ)	β-17-Ketosteroids (mg./24 hr.) Range (mean $\pm 2\sigma$)	β-17-Ketosteroids % of total 17-ketosteroids Mean	β-17-Ketosteroids % of total 17-ketosteroids Standard deviation (σ)	β-17-Ketosteroids % of total 17-ketosteroids Range (mean $\pm 2\sigma$)	Recovery (α+β)/total 17-ketosteroids Mean (%)	Recovery (α+β)/total 17-ketosteroids Probable range (%)	Notes
Baumann & Metzger (1940)	12	M.	13·5	3·4	6·7–20·3	0·9	0·9	0–2·7	6·2	5·2	0–16·6	—	—	(1)*
Frame (1944)	2	M.	9·2	—	—	0·4	—	—	2·8	—	—	97	—	(2)
Salter *et al.* (1946)	12	M.	13·0	3·3	6·4–19·6	1·8	1·1	0–4·0	10·7	7·3	0–25·3	94	74–114	(3)
	12	M.	11·9	3·6	4·7–19·1	1·3	0·5	0·3–2·3	9·5	4·2	0·9–17·9	94	78–110	(4)
Butt, Henly & Morris (this paper)	10	M.	13·6	3·6	6·4–20·8	0·9	0·5	0–1·9	6·4	3·7	0–13·8	95	89–101	(5)
Baumann & Metzger (1940)	4	F.	4·4	—	—	0·2	—	—	3·8	—	—	—	—	(6)*
Frame (1944)	4	F.	6·9	—	—	0·14	—	—	1·75	—	—	92	—	(7)
Salter *et al.* (1946)	13	F.	6·5	2·1	2·3–10·7	1·1	0·9	0–2·9	14·4	11·6	0–37·6	96	63–129	(8)
	14	F.	6·5	2·9	0·7–12·3	1·1	0·7	0–2·5	14·9	8·8	0–32·5	96	62–130	(9)
Butt *et al.* (this paper)	10	F.	13·3	4·3	4·7–21·9	0·7	0·4	0–1·5	4·9	2·1	0·7–9·1	98	92–104	(10)

* These authors do not give figures for a separate determination of 'total 17-ketosteroids'. The sum of the α- and β-fractions has been used in the calculation of the percentage β-17-ketosteroids.

(1) Ages of subjects were from 17 to 49 yr. (av. 26·3). Most of the results obtained on 3-day urine collections.
(2) Subjects 52 and 62 yr.
(3) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944) } On same urine extracts. Subjects aged 20–58 yr. (av. 32·8). Separation into α- and β-fractions carried out by
(4) Antimony trichloride reaction (Pincus, 1943*b*) } method of Baumann & Metzger (1940).
(5) Polarographic method. Subjects aged 20–38 yr. (av. 27·3).
(6) Subjects aged 26–38 yr. (av. 30). Results obtained on 3-day urine collections.
(7) Subjects aged 23–49 yr. (av. 34·5). Results obtained on 3-day urine collections.
(8) *m*-Dinitrobenzene reaction (Cahen & Salter, 1944) } On same urine extracts. Subjects aged 22–54 yr. (av. 30·6). Separation into α- and β-fractions carried out by
(9) Antimony trichloride reaction (Pincus, 1943*b*) } method of Baumann & Metzger (1940).
(10) Polarographic method. Subjects aged 18·5–24 yr. (av. 20·9).

As a further check on the validity of the method, varying quantities of a pooled urine extract were separated into α- and β-fractions by the routine procedure. The results are shown in Table 3. It will be seen that the partition into α- and β-fractions does not appear to be affected by the initial concentration of steroid (cf. the criticism by Salter *et al.* (1946) of the method of Baumann & Metzger (1940)).

The results of the determination of total, α- and β-17-ketosteroids in the urines of ten normal men and ten normal women are shown in Tables 4 and 5. The values quoted for α- and β-17-ketosteroids are the means of duplicate estimations on the same urine extract. The standard deviations among duplicates indicate that reproducibility is satisfactory. It will be seen that the recovery of 17-ketosteroids after fractionation is also satisfactory. In addition it may be noted that the values for total 17-ketosteroids are within the previously published ranges for normal men and women (Barnett, Henly & Morris, 1946; Barnett, Henly, Morris & Warren, 1946).

Although the mean values for the excretion of β-17-ketosteroids are greater for men than for women, both when measured in terms of mg./24 hr. and as percentage of the total ketosteroids, application of Fisher's '*t*' test shows that the differences are not statistically significant.

DISCUSSION

The results of some recent work on the determination of α- and β-17-ketosteroids in normal individuals are collected in Table 6. The data given by the various workers have been used in the calculation of means, standard deviations, etc. In addition the following authors have indicated normal ranges for β-17-ketosteroid excretion without giving individual values: β-fraction usually at least 10–15% of the total 17-ketosteroids (Talbot, Butler & MacLachlan, 1940*b*); β-fraction is 0·6–2·2% of total 17-ketosteroids (Dobriner *et al.* 1942); β-fraction seldom exceeds 15% and is usually 2–3% of the total 17-ketosteroids (Pincus, 1943*a*).

It will be seen from Table 6 that our results for the excretion of α-17-ketosteroids by men are in moderately good agreement with those of other workers. In the case of the women, however, the values found are considerably higher than those found previously. It may be noted that Barnett, Henly, Morris & Warren (1946) found that the polarographic method gave similarly higher results for total 17-ketosteroids, particularly in women.

Our values for β-17-ketosteroid excretion by men show general agreement with those of Baumann & Metzger (1940), but the results obtained by Salter *et al.* (1946) are slightly higher. The corresponding values for women show that the results of Salter *et al.* (1946) are again high, the difference being most marked when the results are expressed as percentage of the total 17-ketosteroids. Some of the normal subjects studied by Salter *et al.* (1946) excreted very large quantities of β-17-ketosteroids. One subject (A.M.) excreted 3·9 mg./24 hr., equivalent to 45·6% of the total 17-ketosteroids, though it may be noted that in this case the sum of the α- and β-17-ketosteroids was some 25% greater than the directly determined value for total 17-ketosteroids (results by *m*-dinitrobenzene reaction).

While each author quoted obtained satisfactory mean recoveries of 17-ketosteroid after fractionation of urine extracts, it is interesting to note the very wide range of recoveries obtained by Salter *et al.* (1946). The reason for this inconsistency is not clear.

Finally, the results described in this paper indicate that the β-17-ketosteroids may contribute up to 15% to the total 17-ketosteroid excretion of normal individuals, though values less than 10% are more usual. No certain sex difference can be demonstrated at the present time. This conclusion is in general agreement with the findings of Baumann & Metzger (1940), Dobriner *et al.* (1942) and Pincus (1943*a*), but is only in partial agreement with the values quoted by Talbot *et al.* (1940*b*).

SUMMARY

1. A micro-method has been described for the quantitative separation of urinary 17-ketosteroid extracts into 3(α)- and 3(β)-hydroxy fractions. The 17-ketosteroids in the separated fractions are estimated polarographically.

2. Recovery of α- and β-17-ketosteroids from pure solutions and from urine extracts is satisfactory.

3. The urinary excretion of α- and β-17-ketosteroids has been determined in a series of ten normal men and ten normal women, and the results have been compared with those obtained by other workers.

4. It is concluded that the total 17-ketosteroids excreted by normal individuals may contain up to 15% of 3(β)-hydroxy compounds, though values less than 10% are more general. There is no significant difference between the β-17-ketosteroid excretion of men and women.

The authors wish to express their thanks to the men and women who co-operated in the collection of 24 hr. urine specimens.

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Studies in Human Mineral Metabolism

1. THE EFFECT OF BREAD RICH IN PHYTATE PHOSPHORUS ON THE METABOLISM OF CERTAIN MINERAL SALTS WITH SPECIAL REFERENCE TO CALCIUM

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The discovery that phytic acid forms relatively insoluble salts with several of the common bases, that are of importance in our diet, has led to several investigations in which the availability of these bases has been determined with varying intakes of phytic acid. The conclusions drawn from such work have been somewhat contradictory: thus, whilst all observers are agreed that the addition of a considerable amount of phytate P is invariably followed by an immediate reduction in the amount of Ca retained, some investigators have suspected, and others are emphatic, that, given time, an adaptation to such a change can occur, and that eventually the body is able to adjust itself fairly satisfactorily to the new conditions.

Obviously laboratory investigations must eventually be reconciled with what happens in everyday life, and a consideration of those human diets which are rich in cereals and hence in phytic acid, yet poor in Ca, lends a considerable amount of support to the view that some such process of adaptation must in fact take place. Otherwise it is difficult to explain how the consumers avoid the disastrous consequences which would arise if part or the whole of this Ca were rendered unavailable.

For two reasons this issue is of particular importance to a country like South Africa. In the first place the large majority of the population, including most of the non-Europeans and not a few of the poorer Europeans, live on just such a diet as has been mentioned above. Secondly, in 1941, a compulsory Standard War Bread, made from 95 to 100% extraction meal, was introduced and is still the only kind of bread allowed to be baked.

As a result of the work published by McCance & Widdowson (1942), the South African National Nutrition Council decided that it was imperative to study the problem under local conditions. For this purpose balance experiments, similar to those described by these workers, were initiated to observe the effects of breads made from high and low extraction meals upon mineral metabolism in adult male subjects.

EXPERIMENTAL

Plan of the experiments. The intake and excretion of Ca, Mg, total P, phytate P and Fe, were determined on four healthy adult European males, over periods ranging from 3 to 19 *consecutive* weeks.

The experimental periods were planned as follows: *A*, from 1 to 2 weeks on the usual everyday diet; *B*, from 4 to 9 weeks on a diet which contained 1 lb. of Standard War Bread (experimental diet no. 1); *C*, from 1 to 4 weeks in which the war bread was replaced by 1 lb. of white bread (70% extraction; experimental diet no. 2). For the first subject only the first two of these periods were observed, as this part of the experiment was of a preliminary nature carried out to gain acquaintance with the procedure.

The balance procedure and the analytical methods employed followed those described by McCance & Widdowson (1942), except in the following details: (i) Tap water was used throughout. (ii) Experience showed that the analytical procedure was facilitated by keeping the food samples separate for the following groups: milk, drinks, fatty foods, eggs, bread, and the remaining foodstuffs, i.e. soup, meat, vegetables, fruit, puddings and so forth. To obtain a thoroughly representative sample this last group was taken to dryness and finely ground. (iii) The total faeces for the week were also taken to dryness and finely ground before sampling.

Subjects. Three of the subjects were research biochemists and the fourth a reliable laboratory technician. During the investigation they lived in their homes and pursued their usual avocations. Health remained excellent in all cases throughout the investigation; no digestive or other disturbances were reported, and fluctuations in weight were insignificant. Some inconvenience was experienced in consuming the required amount of bread, but apart from the tedium inevitably associated with the daily weighing of all foods, etc., no difficulties were encountered.

RESULTS

Phytate-phosphorus content of meal and bread

Analysis of 12 samples of our meal showed that it contained an average of 205 mg. phytate P/100 g. as compared with 214 mg. found for the overseas 92% extraction product. In addition, when samples of bread obtained from bakeries throughout the country were analyzed, it was found that the average figure for 33 loaves was 50 mg. phytate P for 100 g. of fresh bread, as against 'about 100 mg.' found by McCance & Widdowson. Hence our meals were not only no richer in phytate P, but evidently a far greater destruction of this substance had occurred during the baking than in the case of the British 92% extraction loaf.

For the sake of brevity the terms 'brown' and 'white' breads are employed when referring to the 95–100% and the 70% extraction breads respectively.

Results of balance experiments

In view of the recent findings of Steggerda & Mitchell (1946*b*) that faecal Ca may not represent only unabsorbed Ca, attention has been focused solely upon the daily balance of the various elements.

Calcium metabolism

The results are given in Table 1.

A. *Period on the usual diet.* This period was included in order to evaluate the subject's nutritional state before the experimental diets were given. It will be seen that the subjects A.W., L.O. and L.G. were all in slight positive balance.

B. *Experimental diet no.* 1. The subjects were required to consume 1 lb. of Standard War Bread daily, but otherwise they were permitted to eat what they liked, except that the total Ca intake was regulated by adjusting the amount of milk and cheese consumed so as to comply with the requirements based on body weight (10 mg./kg. daily; see p. 458).

Subject F.F. As mentioned above, this study was of a preliminary nature. The subject immediately went into negative Ca balance, which however became less as the experiment proceeded. It was this fact, together with the gradual improvement in the amount of Ca retained, which made us realize the necessity for continuing our observations for prolonged periods. Accordingly it was decided to commence a more lengthy study, during which the degree of adjustment to each change in diet could be followed as quickly as analytical determinations of Ca would permit. In practice it was found possible to be not more than 1 week behind with these determinations. This enabled us to decide when a steady state had been reached, so that the next part of the investigation could be safely proceeded with.

Subject A.W. For no less than 4 weeks this subject remained in continuous negative balance (designated period B_1); this was followed by a further 5 weeks (designated period B_2) during which the balance was continuously positive, or almost positive. If McCance & Widdowson's (1942) suggested limits of significance of ± 25 mg./day are accepted, this subject may be regarded as having reached a state of equilibrium.

Subject L.O. This subject needed 5 weeks to adjust to the new diet (period B_1) and he then remained in equilibrium for the following 3 weeks (period B_2). It must, however, be mentioned that he experienced some difficulty in consuming the full 1 lb. of war bread daily, and hence his intake of other foods tended to suffer. In order to supply the daily requirement of Ca estimated on body weight, a small additional allowance of milk was therefore permitted after the 5th week.

Subject L.G. This subject proved of particular interest to us, since his Ca intake on his usual diet was found to be only just adequate for maintenance. With the experimental diet his Ca intake remained unchanged, and hence, unlike the other subjects, he only had to adjust to the increased ingestion of phytate P. It is presumably for this reason that he

Table 1. *Daily intake, excretion, and retention of calcium and magnesium, from the usual, brown-bread, and white-bread diets*

Period: Duration and sequence	Designation	Diet consumed	(1) Calcium: Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)	(2) Magnesium: Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)
(days)		Subject F.F. Weight 65 kg. Age 50 years										
3	*A*	Usual diet	—	225	914	1139	—	—	98	300	398	—
4	*B*	Experimental diet no. 1 including 1 lb. of standard war bread daily	535	192	504	696	−161	490	108	372	480	+ 10
7	*B*	" " "	501	235	512	747	−246	480	89	395	484	− 4
7	*B*	" " "	490	210	350	560	− 70	535	91	440	531	+ 4
4	*B*	" " "	533	205	390	595	− 62	560	93	450	543	+ 17
		Average figures for period *B*	510	214	437	651	−141	514	94	415	509	+ 5
(weeks)		Subject A.W. Weight 51 kg. Age 31 years										
1	*A*	Usual diet	1160	246	848	1094	+ 66	495	80	405	485	+ 10
2	*A*	"	1175	256	865	1121	+ 54	490	81	415	496	− 6
		Average figures for period *A*	1167	251	856	1107	+ 60	492	80	410	490	+ 2
3	B_1	Experimental diet no. 1 including 1 lb. of standard war bread daily	510	194	402	596	− 86	525	86	450	536	− 11
4	B_1	" " "	512	203	387	590	− 78	550	79	620	699	−149
5	B_1	" " "	524	220	373	593	− 69	575	126	570	696	−121
6	B_1	" " "	501	269	297	566	− 65	575	133	545	678	−103
		Average figures for period B_1	512	221	365	586	− 74	556	106	546	652	− 96
7	B_2	Same as in period B_1	509	225	255	480	+ 29	500	59	430	489	+ 11
8	B_2	" "	515	217	320	537	− 22	585	75	560	635	− 50
9	B_2	" "	509	201	321	522	− 13	575	79	485	564	+ 11
10	B_2	" "	561	219	295	514	+ 47	580	87	475	562	+ 18
11	B_2	" "	488	196	257	453	+ 35	500	77	415	492	+ 8
		Average figures for period B_2	516	212	289	501	+ 15	547	75	473	548	− 1
12	C_1	Experimental diet no. 2*a* including milk, white rolls and potatoes daily	440	170	235	405	+ 35	310	76	225	301	+ 9
13	C_1	" " "	486	145	272	417	+ 69	337	91	235	326	+ 11
14	C_1	" " "	491	183	285	468	+ 23	300	75	210	285	+ 15
15	C_1	" " "	499	170	260	430	+ 69	275	80	185	265	+ 10
		Average figures for period C_1	479	167	263	430	+ 49	305	80	214	294	+ 11
16	C_2	Experimental diet no. 2*b* including 1 lb. of white bread daily	512	245	235	480	+ 32	290	75	195	270	+ 20
17	C_2	" " "	503	260	230	490	+ 13	285	80	195	275	+ 10
18	C_2	" " "	471	200	225	425	+ 46	250	56	190	246	+ 4
19	C_2	" " "	498	155	255	410	+ 88	245	36	195	231	+ 14
		Average figures for period C_2	496	215	236	451	+ 45	268	62	194	256	+ 12

		Average figures for period C_2	496	215	236	451	+ 45	268	62	194	256	+ 12
		Subject L.O. Weight 64 kg. Age 28 years										
1	*A*	Usual diet	1090	139	936	1075	+ 15	512	146	360	506	+ 6
2	*A*	"	927	132	780	912	+ 15	435	92	335	427	+ 8
		Average figures for period *A*	1008	135	858	993	+ 15	473	119	347	466	+ 7
3	B_1	Experimental diet no. 1 including 1 lb. of standard war bread daily	465	117	355	472	− 7	410	145	295	440	− 30
4	B_1	" " "	485	123	375	498	− 13	440	190	322	512	− 72
5	B_1	" " "	506	149	394	543	− 37	480	138	380	518	− 38
6	B_1	" " "	504	155	367	522	− 18	508	130	430	560	− 52
7	B_1	" " "	465	141	450	591	−126	550	101	510	611	− 61
		Average figures for period B_1	485	137	388	525	− 40	478	141	387	528	− 50
8	B_2	Same as in period B_1	620	133	480	613	+ 7	551	110	440	550	+ 1
9	B_2	" "	630	97	520	617	+ 13	482	70	425	495	− 13
10	B_2	" "	660	140	510	650	+ 10	566	120	428	548	+ 18
		Average figures for period B_2	636	123	503	626	+ 10	533	100	431	531	+ 2
11	*C*	Experimental diet no. 2 including 1 lb. of white bread daily	750	182	517	699	+ 51	318	111	202	313	+ 5
12	*C*	" " "	720	170	535	705	+ 15	278	115	166	281	− 3
13	*C*	" " "	695	110	495	605	+ 90	265	102	153	255	+ 10
		Average figures for period *C*	722	154	516	670	+ 52	287	109	174	283	+ 4
		Subject L.G. Weight 75 kg. Age 35 years										
1	*A*	Usual diet	763	193	537	730	+ 33	443	134	335	469	− 26
2	*B*	Experimental diet no. 1 including 1 lb. of standard war bread daily	740	269	550	819	− 79	790	137	584	721	+ 69
3	*B*	" " "	719	220	504	724	− 5	677	172	528	700	− 23
4	*B*	" " "	705	210	485	695	+ 10	735	175	518	693	+ 42
5	*B*	" " "	880	233	595	828	+ 52	755	190	560	750	+ 5
		Average figures for period *B*	761	233	533	766	− 5	739	169	547	716	+ 23
6	*C*	Experimental diet no. 2 including 1 lb. of white bread daily	890	320	535	855	+ 35	465	185	270	455	+ 10

required only 3 weeks to reach equilibrium. After 2 weeks of positive Ca balance it was considered permissible to proceed with the next experimental diet.

C. *Experimental diet no.* 2. *Subject A.W.* Before passing on to the white-bread diet, this subject for a period of 4 weeks was given a diet in which the Ca supplied by the war bread was replaced mainly from milk. The supply of calories was maintained by means of white rolls and extra potatoes (period C_1). The amount of phytate P was thus very considerably reduced, although not as much as might be expected, since white rolls contain more of this substance than white bread made from the same flour. The Ca retention of the first balance period on this diet was the same as that of the last of the previous series. Thereafter the balances became more positive.

After this period of 4 weeks, the subject was placed on a diet in which the rolls and extra potatoes were replaced by 1 lb. white bread (period C_2). It will be seen that the retention of Ca was hardly affected. This period was continued for 4 weeks, and the consistency of the results showed that the subject was well adjusted to the change.

Subjects L.O. and L.G. changed over directly to the white-bread diet without the intervening period C_1. (It is unfortunate that, for reasons beyond our control, both L.O. and L.G. inadvertently consumed slightly more Ca than had been intended.) L.O. showed a slight improvement in Ca retention, while L.G. was virtually in equilibrium in the single balance period in which he was studied.

Magnesium metabolism

The intake of Mg, as will be seen from Table 1, even on the white-bread diet, was always ample for maintenance, i.e. it exceeded 250 mg. daily, the standard suggested by Duckworth & Warnock (1942).

McCance & Widdowson's (1942) subjects were in equilibrium throughout their experiments, but we found, on passing from the usual to the brown-bread diet, that two of our subjects began to lose more Mg than they ingested. As with Ca, however, Mg retention gradually improved. The urinary excretion of Mg was markedly increased during part of the brown-bread-diet period. During the white-bread period, the same total amount was retained as in the previous period, although the intake was only half of that on the brown-bread diet. The possible effect of variations in the Mg intake upon the utilization of Ca is referred to in a later section.

Total phosphorus metabolism

For the sake of brevity the metabolism data for total P and phytate P have been given in Tables 2 and 3 as averages for the different dietetic periods. This course was adopted because the weekly figures during those periods differed little from one another.

The metabolism of total P showed least disturbance of all the elements studied with the changes in diet. Although the amount consumed on the white-bread diet was less than that on the brown-bread diet, the retention rose somewhat. McCance & Widdowson (1942) found the same balance with either type of bread.

Table 2. *The daily intake, excretion, and retention of total phosphorus from the usual, brown-bread, and white-bread diets*

Period			Intake	Urine	Faeces	Total excretion	Balance
Duration	Designation	Diet consumed*	(mg.)	(mg.)	(mg.)	(mg.)	(mg.)
(days)	Subject F.F.*						
3	*A*	Usual diet	—	1520	650	2170	—
22	*B*	Brown-bread diet	885	1435	454	1889	− 4
(weeks)	Subject A.W.*						
2	*A*	Usual diet	1620	1020	542	1562	+ 58
4	B_1	Brown-bread diet	1415	870	529	1399	+ 16
5	B_2	Brown-bread diet	1380	862	483	1345	+ 35
4	C_1	Diet of milk, white rolls and potatoes	1107	760	291	1051	+ 56
4	C_2	White-bread diet	995	685	239	924	+ 71
	Subject L.O.*						
2	*A*	Usual diet	1495	740	752	1492	+ 3
5	B_1	Brown-bread diet	1559	1022	579	1601	− 42
3	B_2	Brown-bread diet	1716	1008	663	1671	+ 45
3	*C*	White-bread diet	1178	705	395	1100	+ 78
	Subject L.G.*						
1	*A*	Usual diet	1600	1000	560	1560	+ 40
4	*B*	Brown-bread diet	1825	1097	769	1866	− 41
1	*C*	White-bread diet	1540	930	500	1430	+110

* For fuller details see Table 1.

Table 3. *The daily intake, excretion, and retention of phytate phosphorus from usual, brown-bread, and white-bread diets*

Period: Duration (days)	Period: Designation	Diet consumed*	Intake (mg.)	Faeces (mg.)	Phytate hydrolyzed (%)
	Subject F.F.*				
3	*A*	Usual diet	—	135	—
22	*B*	Brown-bread diet	425	56	87
(weeks)	Subject A.W.*				
2	*A*	Usual diet	178	65	64
4	B_1	Brown-bread diet	289	33	88
5	B_2	Brown-bread diet	280	24	91
4	C_1	Diet of milk, white rolls and potatoes	84	26	69
4	C_2	White-bread diet	70	6	91
	Subject L.O.*				
2	*A*	Usual diet	142	53	63
5	B_1	Brown-bread diet	289	26	90
3	B_2	Brown-bread diet	345	38	89
3	*C*	White-bread diet	41	22	46
	Subject L.G.*				
1	*A*	Usual diet	105	60	43
4	*B*	Brown-bread diet	315	63	80
1	*C*	White-bread diet	90	50	44

* For fuller details see Table 1.

Table 4. *The daily intake, excretion, and retention of iron, from usual, brown-bread, and white-bread diets*

Period: Duration (days)	Period: Designation	Diet consumed*	Intake (mg.)	Urine (mg.)	Faeces (mg.)	Total excretion (mg.)	Balance (mg.)
	Subject F.F.*						
3	*A*	Usual diet	—	1·0	20·5	21·5	—
22	*B*	Brown-bread diet	26·1	0·4	24·6	25·0	+1·1
(weeks)	Subject A.W.*						
2	*A*	Usual diet	20·5	0·5	19·7	20·2	+0·3
4	B_1	Brown-bread diet	26·0	0·4	25·0	25·4	+0·6
5	B_2	Brown-bread diet	23·9	0·7	22·7	23·4	+0·5
4	C_1	Diet of milk, white rolls and potatoes	14·4	0·2	13·5	13·7	+0·7
4	C_2	White-bread diet	16·0	0·2	15·0	15·2	+0·8
	Subject L.O.*						
2	*A*	Usual diet	14·3	1·1	13·0	14·1	+0·2
5	B_1	Brown-bread diet	19·2	1·3	17·9	19·2	±0·0
3	B_2	Brown-bread diet	23·1	0·9	21·8	22·7	+0·4
3	*C*	White-bread diet	15·1	1·3	13·1	14·4	+0·7
	Subject L.G.*						
1	*A*	Usual diet	18·3	1·2	16·2	17·4	+0·9
4	*B*	Brown-bread diet	25·8	1·4	24·1	25·5	+0·3
1	*C*	White-bread diet	22·7	2·7	19·0	21·7	+1·0

* For fuller details see Table 1.

Phytate-phosphorus metabolism

When the Ca intake was high and that of phytate P comparatively low (i.e. on the usual diet), an average of 59 % of the phytate P was hydrolyzed, whereas when the Ca intake was just adequate for maintenance and phytate-P intake high, an average of 84 % was hydrolyzed. Judging by the conclusions reached by Harrison & Mellanby (1939), one might argue that in the absence of an abundance of Ca, the phytate P forms more readily hydrolyzable salts with bases such as Na and K. The condensed arrangement of the tables, however, masks the interesting fact that, after the change from the usual to the brown-bread diet, the amount of phytate P hydrolyzed reached its maximum extent *immediately*, without the incremental steps of adjustment found for Ca and Mg.

Iron metabolism

Following the suggestion made by McCance & Widdowson (1942), we carried out Fe balances for each subject, mainly because they afforded a simple means of checking the accuracy of the experimental procedure, more especially the marking off of the different faecal periods. The results (Table 4) show that the consumption of brown bread did not affect the amount of Fe retained. Such a conclusion would tend to support the work of several investigators who have found that the consumption of higher extraction breads had no deleterious effect upon the retention of this metal.

DISCUSSION

Environment

When a comparison is made between the experiments of McCance & Widdowson (1942) and our own, it should be pointed out that the present study extended over the different seasons. Owing to our climatic conditions, the subjects not only consumed food which had been fully irradiated by the sun, but were themselves continuously exposed to bright light at an altitude of approximately 6000 feet. There was thus this difference between the two studies, but, as shown by the very full review given by McCance & Widdowson, there is little evidence to suggest that the addition of vitamin D to the diet of healthy adults has any appreciable effect upon the absorption of Ca.

Calcium balances

At the outset we desire to draw attention to certain sources of error inherent in balance studies of this particular kind, which do not always appear to be sufficiently appreciated.

In recent years evidence has been accumulating that, when an individual who is habituated to one level of Ca intake is suddenly placed on a lower level, a period of adjustment is inevitable and may even be somewhat prolonged (Nicholls & Nimalasuriya, 1939; Cathcart, 1940; Steggerda & Mitchell, 1941; Kraut & Wecker, 1943). According to these workers the time taken to reach a steady state at the new level of Ca intake depends upon the magnitude of the difference between the two levels. It must also depend upon the extent to which the new supplies of Ca are available. Thus, if the reduction of the Ca intake is accompanied by an increase in the phytate-P intake, the negative balances following such changes cannot be interpreted as being solely due to the interfering effects of the phytate P.

Also, when passing from a high to a lower Ca intake it is necessary to insure that at least enough Ca is provided to satisfy the minimum requirements of the individual concerned. Otherwise the negative balances that will be observed are likely to be due to what may be termed an 'excretion momentum', in addition to any effects arising from a change in the availability of the supply. The difficult question of what constitutes a minimum daily requirement of Ca has recently been the subject of intensive study by Leitch (1937), Holmes (1945) and more especially Mitchell and his associates (Mitchell & Curzon, 1939; Steggerda & Mitchell, 1939, 1941, 1946*a*). These workers have reached substantial agreement with a figure of about 10 mg./kg. body weight, and we accordingly adopted this standard when planning our experiments.

Furthermore, in the reports given by some investigators the experimental periods are interrupted by days or weeks during which the food consumed is not specified. Unless the same level of Ca intake is maintained it seems inevitable that such interruptions will affect the subsequent observations. Lastly, there are other workers who employ such short experimental periods, and change the diets so frequently, that it is questionable whether the body has time to adjust itself to one diet before the next is taken. An extreme example of this is to be seen in the study reported by Wang, Liu, Chu, Yu, Chao & Hsu (1944), where, within the space of 38 days the diet was changed no less than twelve times.

The assimilation of calcium in the presence of phytic acid

Although our main experiments were carried out on only three subjects, the results obtained confirmed one another.

It is not easy to compare our findings with those reported by other observers because of the comparatively short experimental periods usually employed by them. Thus, whilst the experiments reported by McCance & Widdowson (1942) lasted intermittently over 9 months, the actual periods of continuous observation were usually 3–4 weeks. These workers were emphatic that there was no indication of any adaptation occurring. Similarly, the experimental periods employed by Krebs & Mellanby (1943) lasted for 4 weeks; but it is worth noting that before the experiments on the 85% extraction bread terminated, one of their six volunteers (no. 3) was already showing improvement in the retention of Ca and finally reached equilibrium. In their recent experiments with children, Hoff-Jørgensen, Andersen & Nielsen (1946) suspected that the retention of Ca was gradually improving with time. (We presume, although this is not stated, that the same diet, rich in phytic acid, was continued during the short intervals that occurred between their 5-day periods.) Indeed, had we discontinued our observations after 3–4 weeks on the high-extraction bread diet, our findings would have been

very similar to those obtained by these three groups of investigators.

Combining our own evidence with that already available, we suggest the following: subjects passing abruptly from their usual diet to one containing less Ca and much more phytate P show an immediate negative Ca balance. As the body becomes accustomed to such a diet, the retention of Ca improves, so that, *given time*, equilibrium again occurs and the losses of Ca are slowly made good. The period of adjustment is shorter, and the loss of Ca is less, if the disparity between the usual Ca intake and that of the experimental diet is small.

Evidence available from studies made on individuals habituated to diets high in phytate phosphorus and low in calcium.

There is, however, another method of approach which throws valuable light on the whole problem. Instead of placing a few selected individuals on prescribed diets and then observing how they adjust themselves to the new conditions over shorter or longer periods (which for obvious practical reasons can never be really very prolonged relative to the life span), it ought to be possible to gain more reliable information by studying people who, through force of circumstances, are compelled to subsist for long periods on diets which are very rich in phytate P and very low in Ca. Some of the few available Ca balance studies that are on record for such people have been assembled in Table 5.

Although we have deliberately selected examples where the Ca balances were positive, it may be stated that this obtained in all the cases reported by Nicholls & Nimalasuriya (1939); in two out of the five cases reported by Henderson & Kelly (1929–30) for boys on prison diet; in two out of four adult cases on the same type of diet (Kelly & Henderson, 1929–30); and in two out of the three subjects studied by Basu, Basak, & Sircar (1939). This must presumably indicate that such positive balances occur fairly frequently. Even the survival of these individuals on such diets is remarkable. That they are somewhat shorter and lighter than well nourished Europeans is admitted, but with a diet lacking in so many other respects it is hardly fair to attribute this solely to a lack of available Ca. As far as our rural South African Bantu are concerned there is not much evidence of poor skeletal calcification, whilst the teeth are often well formed and sometimes remarkably free from caries. Thus in a recent careful dental survey carried out on some 1000 Bechuanaland children, whose diet consists mainly of Kaffir corn, no less than 70% were found to be free from all signs of caries. Equally striking results have been reported by Wilson & Widdowson (1942) for Indian children.

It must be admitted that such observations cannot easily be reconciled with orthodox opinions; however, we do not consider it satisfactory to dispose of them as being due to racial differences, but think rather that they reflect an adaptation which any human being may be capable of making under similar circumstances. That somewhat similar adaptation can be achieved by Europeans is suggested by the experience of the French people during the occupation. They consumed a diet extremely low in Ca and including high-extraction bread (Paris

Table 5. *Examples of positive calcium balances observed with non-Europeans of different races when consuming their typical cereal diets, rich in phytate phosphorus and low in calcium*

Diet and authority	Sex and age (yr.)	Experimental period (days)	Intake (mg./day)	Urine (mg./day)	Faeces (mg./day)	Total excretion (mg./day)	Balance (mg./day)
(1) Nicholls & Nimalasuriya (1939). Sinhgalese children consuming usual diet of cereals, legumes, roots and vegetables	*M* 4	3	205	25	55	80	+125
	M 4	3	183	24	96	120	+ 63
	F 7	3	245	29	135	164	+ 81
	M 7	3	70	4	15	19	+ 51
	M 7	3	223	7	47	54	+169
	M 7	3	220	5	45	50	+170
(2) Henderson & Kelly (1929–30). Bantu boys consuming long term prison diet including 1·5 lb. maize, beans, potatoes, fat and meat. Average of two 4-day periods taken during a 21-day study	*M* 16	8	300	23	271	294	+ 6
	M 16	8	300	19	242	261	+ 39
(3) Kelly & Henderson (1929–30). Bantu adults consuming diet almost the same as in (2). Average of a 4-day period taken once during a 42-day study. Prison diets (2) and (3) both resembled diets commonly consumed	*M*	4	300	23	151	174	+126
	M	4	300	10	172	182	+118
(4) Basu *et al.* (1939). Indian adults. Diet (*a*) included 400 g. rice; average of 6-day period. Diet (*b*) included 600 g. whole wheat: average of 12 consecutive days	(*a*) *M*	6	280	70	162	232	+ 48
	(*b*) *M*	12	310	19	167	186	+124

letter, 1942), but it is claimed that the decalcification which might have been expected did not take place (Paris letter, 1946).

It is possible that some similar process of adaptation had occurred, though to a lesser extent, in our subjects, since for several years the inhabitants of South Africa have been restricted solely to the use of high extraction bread, and hence some degree of habituation to it may have occurred. This capacity for adjustment may well have been lacking in the subjects studied by McCance & Widdowson (1942).

The mechanism of adaptation to diets with a high phytic acid content

This adaptation can be explained in two ways: one based on our knowledge of the chemical changes which phytates may undergo in the intestine, the other on the body's known ability to adapt itself to a lower intake of Ca.

(*a*) *The digestion of phytate phosphorus.* Most workers seem to agree that phytate P and Ca combine to form an insoluble salt in the intestine. Thus Harrison & Mellanby (1939) from their work on puppies remark: 'It is not possible to say whether such actual precipitation occurs under the conditions present in the gut, but it seems not unlikely.' McCance (1946), discussing the position as far as humans are concerned, concludes that phytic acid 'precipitates Ca in the intestine and by so doing prevents its absorption'. Assuming that the pH values in the intestinal tract of man resemble those observed in the pig, Møllgaard, Lorenzen, Hansen & Christensen (1946) conclude that 'phytic acid may precipitate Ca even in the first part of the small intestine thus causing a serious fall in its absorption'. Hence the consensus of opinion is definitely that the precipitation of Ca by phytic acid occurs, and the fact must therefore be accounted for that much of this Ca is eventually absorbed.

There is general agreement that some degree of hydrolysis of phytate P occurs in the digestive tract. This has been demonstrated for pigs, rats, dogs and hens. Similar results for man have been reported by McCance & Widdowson (1935), Wang *et al.* (1944), Cruickshank, Duckworth, Kosterlitz & Warnock (1945), and recently for babies and children by Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) and Hoff-Jørgensen, Andersen & Nielsen (1946).

As far as animals are concerned, the extent to which hydrolysis occurs appears to depend on the level of the Ca intake, e.g. for rats, hens and dogs it has been found that the lower the Ca intake the greater the amount of phytate P hydrolyzed. Likewise for man Cruickshank *et al.* (1945), in a study in which the cereal employed was oatmeal, found that the amount of phytate P hydrolyzed was inversely proportional to the Ca intake. Similar results are reported in the present paper.

At present there are two views as to how this hydrolysis is brought about in man. Some maintain that it is due to the action of the intestinal flora in the digestive tract, whilst Møllgaard *et al.* (1946) insist that hydrolysis can only occur when the diet contains a specific enzyme, which they regard as being normally destroyed when food is cooked.

Both in the case of animals and man there seems to be no difference of opinion that at least a proportion of the P liberated by hydrolysis is absorbed. The question at issue is whether hydrolysis occurs at such a level as will also permit of the liberated Ca being absorbed.

It has been shown that under certain circumstances this does take place in the case of pigs, rats, hens and dogs, and it is of interest to note that Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) conclude from their experiments on children 'that a small part of the Ca which was precipitated as Ca phytate was absorbed'. Similarly, Wilson & Widdowson (1942) reviewing their Indian observations, where they were dealing with diets which contained a great deal more phytic acid P than was sufficient to precipitate all the Ca present, came to the same conclusion.

A further interesting point arises from a consideration of the part Mg may play in this connexion. It is more than likely that the phytate P precipitates in the intestinal tract a compound which is not solely the Ca salt, as seems to be assumed by Hoff-Jørgensen (1946), Hoff-Jørgensen, Andersen, Begtrup & Nielsen (1946) and Hoff-Jørgensen, Andersen & Nielsen (1946). Both Harrison & Mellanby (1939) and McCance & Widdowson (1942) have shown that when precipitation occurs *in vitro* in the presence of both bases, a mixed phytate is formed containing approximately equal equivalents of Ca and Mg. The latter workers, who attempted to simulate the conditions which occur in the intestinal tract in regard to pH value, are of the opinion that a precipitate of this nature is likely to be formed. Obviously this is an important point, for it would decide the amount of Ca made less available by a given amount of phytate P. Our own results tend to support the proposition that phytic acid reacted with Mg as well as with Ca.

(*b*) *The ability of the body to adapt itself to a lowered calcium intake.* Irrespective of the composition of the diet, it is now well known that the body can adapt itself to a Ca intake often well below 10 mg./kg. body weight daily. This has been stressed by Nicholls & Nimalasuriya (1939), Owen, Irving & Lyall (1940), Steggerda & Mitchell (1941, 1946*a*) and Kraut & Wecker (1943). This adaptation, in the present experiments, was not due only to increased hydrolysis of phytate, since the increased hydrolysis took place immediately the brown-bread regime was instituted, whereas the adaptation was gradual.

The ability of the body in this respect has been well summarized by Steggerda & Mitchell (1941) as follows: 'It appears that in the presence of an inadequate supply of any nutrient, including Ca, the body can adjust itself to the situation, either by a more economical use of what little is available, or by a lowering of its own requirements, so that eventually it comes into equilibrium with the limited food supply.' These writers (Steggerda & Mitchell, 1946*a*) consider that no harm can come from such an adaptation, provided the Ca intake is 10 mg./kg. body weight daily.

This work was originally undertaken to find out if the results of McCance & Widdowson (1942) applied in South Africa. It is evident that our results confirm those of these workers in so far as we have shown that a high-brown-bread regime renders a certain amount of the dietary Ca less available. However, further evidence shows beyond doubt that the body can adjust to this lessened availability, and that the consumption of such a diet over long periods has no deleterious effect upon Ca metabolism.

SUMMARY

1. The metabolism of calcium, magnesium, phosphorus, phytate phosphorus, and iron has been followed in three healthy adult European males for periods lasting from 7 to 19 *consecutive* weeks. During this time the subjects consumed (*a*) their usual diet, (*b*) a diet including 1 lb. of bread made from 95 to 100% extraction meal, and (*c*) a diet including 1 lb. of bread made from 70% extraction meal. During dietary periods (*b*) and (*c*) the daily calcium intake was restricted to a level of approximately 10 mg./kg. of the subject's body weight.

2. When the subjects passed abruptly from their usual diet to one containing much more phytate phosphorus and, in two cases, less calcium, they showed an immediate negative calcium balance.

3. As they became accustomed to such a change, the retention of calcium improved, so that, *given time*, equilibrium was again reached and the losses of calcium were slowly made good.

4. The period of adjustment was shorter, and the loss of calcium was less, if the disparity between the usual calcium intake and that from the experimental diet was small.

5. The lower the calcium content of the diet, the greater was the amount of phytate phosphorus hydrolyzed.

6. The results are tentatively explained as being due to hydrolysis of the calcium and magnesium phytate at such a level in the digestive tract as to permit of the calcium being subsequently absorbed, and to a gradual adaptation of the body to a lowered intake of available calcium.

7. In two of the subjects, the retention of magnesium was at first lowered by the diet containing more phytate phosphorus. It was considered that this might be due to the precipitation of magnesium by the phytic acid. Later, while the subjects were still on this regime, magnesium retention improved, and changed but slightly when the phytate phosphorus of the diet was lowered.

8. The retention of total phosphorus was at first lowered in two of the subjects by the addition of phytate phosphorus to the diet, but subsequently improved. It improved still further in all subjects when the phytate phosphorus of the diet was reduced.

9. The retention of iron was virtually the same with high and low phytate-phosphorus diets.

10. Cereal diets rich in phytate phosphorus and low in calcium are commonly consumed in various parts of the world; consideration of what must occur under these conditions, together with an examination of the small amount of relevant experimental evidence available, lends a considerable amount of support to the conclusions reached in this paper.

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β-Glucuronidase and Cell Proliferation*

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After repeated feeding of menthol to mice, Fishman (1940) obtained results which, on statistical examination, showed an increase in β-glucuronidase activity in liver, spleen and kidney, as compared with organs from untreated animals. Similar results were obtained in dogs fed with borneol. Glucuronidase in uterus and other sex organs was unaffected by menthol and borneol. In Fishman's own interpretation of these important experiments, β-glucuronidase is assumed to be responsible for glucuronide synthesis in the body. A synthetic role for the enzyme has, however, still to be demonstrated, its physical properties and distribution in the body having been studied solely by means of its hydrolytic action on conjugated glucuronides. Since menthol and borneol have been proved to be excreted as the glucuronides in, e.g. the dog, and may conceivably behave in the same way in the mouse, Fishman suggested that in his experiments he was measuring adaptation by glucuronidase in response to the presence of excess substrate for its hypothetical synthetic action. Later this theory was extended to explain the elevation in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947). Oestrogens did not affect the enzyme in liver, spleen and kidney, and the additional assumption was required, and made, that the enzyme is specific in its synthetic action, according to its source, for different groups of substrate. No such specificity was, however, observed in its hydrolytic action *in vitro*, menthol glucuronide being used throughout in the assay of uterine glucuronidase under conditions found to be optimal for hydrolysis by spleen preparations.

Fishman determined the activity of his enzyme extracts by measuring, by means of its reducing power, glucuronic acid liberated from menthol glucuronide (Fishman, 1939). Sources of error in this procedure, arising largely from its lack of specificity, have been pointed out by other authors (Graham, 1946; Levvy, 1946, 1948), and have led to the development of more satisfactory methods of assay (Talalay, Fishman & Huggins, 1946; Kerr, Graham & Levvy, 1948).

Using phenol glucuronide as substrate in the assay of glucuronidase (Kerr *et al.* 1948), an attempt was made to confirm Fishman's findings (1940) with menthol. Within 24 hr. of a single intraperitoneal injection of L-menthol into mice, there was a marked rise in glucuronidase activity in liver, but not in spleen and kidney. Liver damage was observed and confirmed histologically, and it was subsequently shown that a rise in β-glucuronidase in liver or kidney, depending upon the organ or organs attacked, followed administration of a variety of toxic agents to mice. A more extensive examination of the action of menthol revealed, in addition to the effect on liver, delayed damage to kidney, followed by an increase in glucuronidase activity in this organ also. An increase in the glucuronidase activity of an organ was found, in general, to be associated with active cell proliferation provoked by injury, rather than with the injury itself, and high values were seen in the livers of adult mice after sub-total hepatectomy, and in the liver, spleen and kidneys of infant mice.

* Preliminary accounts of parts of this work have been published elsewhere (Kerr & Levvy, 1947; Kerr, Levvy & Campbell, 1947), and the principal findings were described in a paper read to the Biochemical Society on 27 September 1947 (Levvy, Kerr & Campbell, 1948).

EXPERIMENTAL AND RESULTS

Enzyme assay. In the assay of kidney glucuronidase it was assumed that the conditions for optimum hydrolysis of phenol β-D-glucuronide would be the same as those previously found to hold for spleen and liver preparations (Kerr *et al.* 1948). All preparations of the enzyme were diluted to final volumes giving readings of 20–40 μg. phenol in the assay, after correction for blanks. The results are shown in the tables and figures in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015M-phenol glucuronide at 38° and pH 5·2. The standard error is given wherever possible. Although frequently based upon too small a group of animals to have any statistical value, it shows the variation in the individual figures in a convenient form.

Histology. Portions of organs from animals used for enzyme assay, or whole organs from other animals treated similarly, were fixed immediately in Susa and taken in the usual way through the ethanols to a mixture of chloroform and cedarwood oil, and finally cleared in pure cedarwood oil. After embedding in paraffin wax, sections were cut at 8 μ. and stained with Mayer's haematoxylin and eosin. The distribution of fat was studied in frozen sections, prepared from tissues rapidly fixed by heat in formol saline, and stained with haematoxylin and Sudan III.

Damage, repair and cell division are shown in the tables by an arbitrary system of + signs. In the case of damage, + indicates that while definite it was neither severe nor extensive, and + + + that it was at its greatest for the toxic agent in question. The course of repair is measured likewise, + + + indicating that replacement of damaged tissue by normal cells is practically complete. Under cell division, an estimate is given of the number of mitotic and amitotic figures and hyperchromatic nuclei *in excess of normal*. No histological findings are given for spleen since deviations from normal could never be distinguished in this organ.

Normal mice and vehicle controls. Average values for β-glucuronidase in each organ were the same for normal adult mice (30–40 g.) of both sexes and drawn from three different colonies, and all the results are grouped together in the tables. Spleen showed greater variation in its normal glucuronidase activity than did liver or kidney. Intraperitoneal injection of relatively large volumes of 0·9 % sodium chloride solution, olive oil or nut oil (the vehicles used for administration of toxic agents) had no effect on glucuronidase in any of the three organs examined after an interval of 1–2 days. These results are not shown. The relatively small number of experiments in which nut oil was used as a vehicle, olive oil being unobtainable, are included in the tables with those done with the latter as medium.

Effects produced by a single injection of L-*menthol.* Intraperitoneal injection of L-menthol (Table 1) caused a rapid rise in liver glucuronidase activity, reaching a maximum after 24 hr. and persisting for 7 days. Greatest liver damage was observed after 24 hr., but repair processes were not perceptible at this time. After 14 days, repair was almost complete and the enzyme level had returned to its original value, although cell division still seemed to be slightly in excess of normal. In the first 24–48 hr., kidney was normal in structure and in its enzyme activity, but after 3 days damage was evident and the figure for glucuronidase had risen after 7 days. At the end of 14 days this organ was normal in all respects. No effect of menthol on spleen glucuronidase was observed at any stage. Sex did not influence the results obtained with liver and kidney.

Mills (1947) found beef spleen glucuronidase to consist of two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide, and the pH activity curves for hydrolysis of phenol glucuronide by enzyme from mouse spleen and liver (Kerr *et al.* 1948) had subsidiary peaks at pH 4·5. An experiment was done in which spleen, liver and kidney glucuronidase activities, 24 hr. after injection of menthol, were compared with normal at pH 4·5 instead of 5·2. The change in pH had no appreciable effect on the results compared with those shown in Table 1.

No details of the toxic action of menthol could be found in the literature. A brief description of the changes seen in liver and kidney may be of interest. In the liver, the first deviation from normal was cloudy swelling, followed by fatty change and necrosis surrounding the central vein and extending about a third of the way into the lobule. The nuclei showed hypertrophy and hyperchromatism, many

Table 1. *Changes in β-glucuronidase and histological findings after injection of mice with* L-*menthol*

(333 mg. Menthol/kg. injected intraperitoneally in olive oil. Average enzyme activity and standard error expressed as G.U./g. moist tissue (see text). Number of animals in group shown by figures in brackets.)

Interval (days)	Spleen enzyme	Liver				Kidney			
		Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
Untreated	636±70 (23)	273±13 (23)	—	—	—	363±24 (11)	—	—	—
0·125–0·5	720±63 (9)	467±24 (9)	—	—	—	381±40 (9)	—	—	—
1	690±41 (3)	823±135 (3)	+ + +	0	0	285±46 (3)	0	0	0
2	738±86 (3)	884±74 (6)	—	—	—	344±71 (3)	—	—	—
3	903±208 (3)	953±39 (3)	+ +	+ + +	+	—	+ +	0	0
7	646±86 (6)	775±46 (7)	+	+ +	+ +	603±52 (7)	0	+	+ + +
14	600±14 (3)	318±17 (3)	+	+	+ +	337±23 (3)	0	0	+ + +

binucleate cells appeared (amitotic division), and at a later stage mitotic division became evident. The Küpffer endothelial cells were swollen. In the case of kidney, the damage was not severe, being confined to the distal portions of the convoluted tubules and to some glomeruli, the endothelium of which was swollen and in places necrotic. Intraperitoneal injection of mice with large doses of menthol (about 0·7 g./kg.) caused prolonged depression of the respiration and unconsciousness. No attempt was made to determine the lethal dose.

Repeated administration of L-*menthol and* L-*menthol β-*D-*glucuronide.* Results for β-glucuronidase in spleen, liver and kidney, after intraperitoneal injection of mice with L-menthol or its glucuronide twice or thrice daily for varying periods (Table 2), were similar to those obtained after a single injection of L-menthol. The glucuronide was injected as a neutral solution in 0·9 % sodium chloride solution and menthol itself as a solution in olive oil. (For the preparation of neutral solutions of acid compounds in 0·9 % sodium chloride solution, see Chance, Crawford & Levvy, 1945.) Repeated oral administration (Odell, Skill & Marrian, 1937) of menthol produced an increase in liver glucuronidase activity of the same order as the injections, except in mice receiving a total dose of 9·3 g./kg. in which the rise was barely perceptible. The latter was, however, as great as that obtained by Fishman (1940) and proved to be statistically significant ($P = 0·01$). This experiment was carried out exactly as described by Fishman except that three of the mice were given a solution of menthol in olive oil instead of an emulsion in soap solution. The change in vehicle had no effect on the response of the enzyme, and only solutions in oil were used in the other feeding experiments (total dose 1·2 and 2·0 g./kg.). No histology was done in the experiments listed in Table 2, except in the case of menthol glucuronide, which produced changes similar to those seen after a single injection of menthol.

Changes produced by single injections of a variety of substances. The changes in β-glucuronidase activity and histological findings in liver and kidney after injection of various substances, some of them known liver or kidney poisons, are summarized in Table 3. Spleen was also examined in these experiments. Since any changes in glucuronidase in this organ were relatively small, with wide variation in individual figures, the results are not shown.

Subcutaneous injection of carbon tetrachloride in olive oil caused severe fatty degeneration and early necrosis in liver within 24 hr. After 3 days, damage was extensive, but repair processes had commenced, and after 7 days repair was far advanced. A marked increase in liver glucuronidase activity occurred within 24 hr., and this was maintained for 7 days.

Table 2. *Changes in β-glucuronidase after repeated administration of* L-*menthol and* L-*menthol β-*D-*glucuronide*

(Results expressed as in Table 1)

Agent and mode of administration	Total dose (g./kg.)	Interval after 1st administration (days)	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Untreated	—	—	636±70 (23)	273±13 (23)	363±24 (11)
L-Menthol, orally	1·2	3	843±185 (3)	741±146 (3)	—
	2·0	1	—	895±77 (6)	—
	9·3	5	499±48 (6)	369±40 (6)	260±2 (2)
L-Menthol, intraperitoneally	0·8	2	254±38 (3)	1149±136 (3)	—
	1·2	3	599±42 (6)	869±58 (6)	—
L-Menthol β-D-glucuronide, intraperitoneally	2·3	1·5	995±95 (3)	1104±222 (3)	295±13 (3)

There were no marked pathological changes in kidney at any stage, nor was there any rise in glucuronidase in this organ. Intraperitoneal injection of carbon tetrachloride (0·5–2 g./kg.) produced a change in liver glucuronidase similar to that already described for subcutaneous injection.

Mercuric nitrate given subcutaneously in 0·9 % sodium chloride solution had no very marked effect on liver, but produced severe cortical necrosis with hyaline casts in kidney within 24 hr. Kidney glucuronidase activity showed no rise at this stage, but after 3 days, by which time repair was practically complete, it was more than twice its normal value.

The changes in liver after subcutaneous injection of chloroform in olive oil resembled those produced by carbon tetrachloride. Kidney, however, showed an interesting sex specificity in the response of the enzyme to chloroform. In agreement with the observation of Eschenbrenner (1944), this compound was found to cause renal necrosis in male, but not in female mice. The rise in kidney glucuronidase activity, which was confined to male mice, was not seen in the early stages of the damage, but was evident after 8 days, by which time repair was extensive. An increase in liver glucuronidase

Table 3. *Effects of various agents on liver and kidney*

(Results expressed as in Table 1)

Agent and dose	Interval (days)	Sex	Liver				Kidney			
			Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
None	—	♀, ♂	273±13 (23)	—	—	—	363±24 (11)	—	—	—
Carbon tetrachloride (5·3 g./kg.)	1	♂	1138±148 (3)	+++	0	0	139±9 (3)	+ (?)	0	0
	7	♂	927±48 (4)	+	+++	++	323±45 (4)	0	0	0
$Hg(NO_3)_2$ (20 mg./kg.)	1	♂	436±28 (2)	+	0	0	208±42 (3)	+++	0	0
	3	♂	469±29 (5)	0	+	0	808±61 (5)	0	+	+++
Chloroform (2 g./kg.)	1	♂	939±90 (7)	+++	++	0	194±16 (6)	+++	0	0
	8	♂	711±34 (3)	+	++	++	628±119 (3)	+	++	++
	1	♀	583±27* (3)	—	—	—	251±18 (3)	0	0	0
	8	♀	608±44 (3)	+	++	++	274±15 (3)	0	0	0
Yellow phosphorus (7·5 mg./kg.)	2	♂	91±23 (3)	+++	0	0	338±35 (3)	+ (?)	0	0
	5	♂	744±37 (3)	++	++	+	462±89 (3)	0	0	0
	10	♂	429±80 (3)	0	0	+++	309±32 (3)	0	0	0
Sulphathiazole (43 g./kg.)	3	♂	460±29 (3)	+	0	0	368±13 (3)	0	0	0
Pregnanediol (333 mg./kg.)	1·7	♀	287±65 (3)	0	0	0	321±64 (3)	0	0	0
†Pregnanediol β-D-glucuronide (800 mg./kg.)	1·7	♀	241±36 (3)	—	—	—	264±19 (3)	—	—	—
Ether (40 min. deep anaesthesia)	1·75	♀, ♂	265±30 (4)	0	0	0	309±63 (3)	0	0	0
Sodium sulphapyridine monohydrate (18–36 g./kg.)	2	♀, ♂	327±17 (7)	++	+	+	362±11 (7)	+ (?)	0	0

* Results for glucuronidase in liver obtained after intraperitoneal injection of 0·5 g. chloroform/kg.

† Pregnane-3(α):20(α)-diol glucuronidic acid free from pregnane-3(α)-ol-20-one glucuronidic acid (Sutherland & Marrian, 1947).

activity was observed after injection of as little as 0·2 g. chloroform/kg. subcutaneously.

Yellow phosphorus, injected subcutaneously in olive oil, had no marked effect on kidney, but produced profound and extensive changes in liver (congestion, fatty degeneration and necrosis). From the results of experiments dealt with above it will have been noted that there may be no rise in glucuronidase activity in an organ when damage is at its height. In the case of phosphorus, there was an unmistakable initial drop in the activity of the enzyme, to one-third of its normal value. When, at the end of 5 days, repair was well under way, the enzyme level showed the usual increase, only to fall again when repair was complete.

Of the remaining substances listed in Table 3, ether and pregnanediol produced no pathological changes and had no effects on glucuronidase in either liver or kidney. Pregnanediol glucuronide resembled the parent compound in its effects on the enzyme, but was not examined for histological effects. Ether was given by inhalation, and pregnanediol and its glucuronide were injected intraperitoneally as suspensions in olive oil. Sulphathiazole caused cloudy swelling in liver after subcutaneous injection of a very large dose as a neutral solution in 0·9% sodium chloride solution. There was a small, but significant ($P = 0·05–0·02$) rise in liver glucuronidase activity. This compound had no effect of any kind on kidney. Sodium sulphapyridine, given in the same way as sulphathiazole, caused fatty degeneration and necrosis in liver, accompanied by some cell proliferation, but without appreciable change in the enzyme level. In some animals there was slight damage to the kidneys, again without any rise in glucuronidase activity.

Uranyl acetate. Results for glucuronidase in liver and kidney after subcutaneous injection of mice with varying doses of uranyl acetate in 0·9% sodium chloride solution (Fig. 1) illustrate the point that increasing the dose of a toxic agent may retard the rise in glucuronidase activity in the early stages of poisoning, and may even cause an initial drop in the enzyme level. Each point in the figure is an average for a group of three male mice, killed 2 days after injection. Severe tubular 'nephrosis' was noted at this stage with all four doses of the toxic agent. Cell proliferation could also be seen after all but the

largest dose, becoming more marked as the dose fell. In the case of liver, the histological findings were more difficult to interpret as the damage, which was mainly subcapsular, was transitory and rapidly succeeded by intensely active cell proliferation. Only the latter response was observed after injection of the smallest dose of uranyl acetate. In general, however, damage was greater and repair processes advanced after 5 days. At the end of 10 days, kidney was entirely normal and liver repair was almost complete. With the smaller dose (0·2 mg./kg.), cell proliferation was marked in both organs after 1 day, and repair was far advanced after 4 days. Phenylarsenoxide had no effect on spleen glucuronidase, and the histological changes produced in liver and kidney were similar for both sexes.

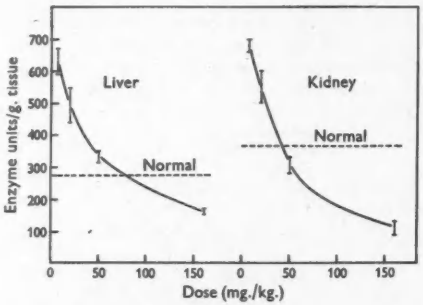

Fig. 1. Liver and kidney glucuronidase activity 2 days after subcutaneous injection of mice with varying doses of uranyl acetate. Mean ± S.E. shown for each point.

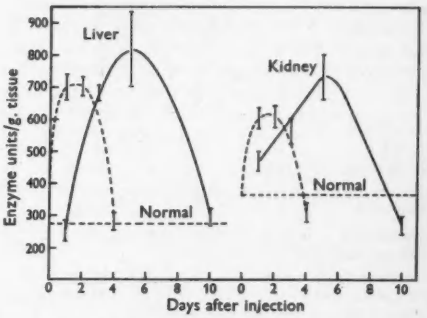

Fig. 2. Liver and kidney glucuronidase activity at varying periods after subcutaneous injection of mice with phenylarsenoxide. ——, 1 mg./kg.; — —, 0·2 mg./kg. Mean ± S.E. shown for each point.

slower to appear as the dose was increased. Ten days after injection of the smallest dose of uranyl acetate repair was finished in both liver and kidney, and their enzyme levels had returned to normal. No change in spleen glucuronidase was produced by uranyl acetate.

The possibility that in a severely damaged organ an apparently normal value for glucuronidase may be observed at a certain stage, even though cell proliferation may have commenced, probably explains the fact that only small rises in the liver enzyme were observed after prolonged feeding of menthol (total dose 9·3 g./kg., Table 2) or subcutaneous injection of sodium sulphapyridine (Table 3).

Phenylarsenoxide. Results obtained with this compound (Fig. 2) show the changes in liver and kidney glucuronidase activity at various stages in different degrees of poisoning. Phenylarsenoxide was injected subcutaneously as a neutral solution in 0·9 % sodium chloride solution. Each point in the figure is an average for a group of three male mice, except in the case of the 1 day figures with the larger dose, which are both based on six results. Phenylarsenoxide caused peripheral lobular necrosis and fatty degeneration in liver, and diffuse nephritis in kidney. Damage to both organs was intense 1 day after injection of 1 mg./kg., with no signs of repair. After 3 days, repair processes had become evident, and replacement of damaged tissue was well

Effects of various substances on the enzyme in vitro. All the substances examined for their effects on β-glucuronidase activity *in vivo*, with the exception of pregnanediol glucuronide, were tested for their effect on the assay *in vitro* in a concentration of 0·1 % (w/v). A solution in the medium used for injection was added to the citrate buffer and shaken vigorously. In no case did the presence of the agent in the incubation mixture affect the activity of β-glucuronidase from normal mice.

Infant mice and partially hepatectomized mice. As shown in Table 4, glucuronidase activity in spleen, liver and kidney was much higher in young mice, ranging in age from 1 to 15 days, than in normal adults. The remaining lobes of liver in adult mice (male and female), 3–8 days after sub-total hepatectomy, were hypertrophied. The glucuronidase level was high and cell proliferation was very active. In preparing the animals, 60 % of the liver was removed by cautery or ligature under ether anaesthesia, the whole operation taking less than 10 min. There was no difference in the final result between the alternative surgical techniques, nor at the various times of examination. One animal (result omitted from Table 4) was comatose and apparently about to expire when killed 3 days after operation. As expected, the remaining fraction of the liver showed no increase in weight, in the glucuronidase activity nor in cell proliferation.

Table 4. *β-Glucuronidase after sub-total hepatectomy and in infant mice*

(Results expressed as in Table 1)

Age (days)	Treatment	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Adult	None	636±70 (23)	273±13 (23)	363±24 (11)
1*	None	5100 (3)	1370 (3)	881 (3)
5*	None	2670 (2)	1294 (2)	702 (2)
5*	None	3820 (2)	1432 (2)	883 (2)
13*	None	1521 (2)	2218 (2)	606 (2)
15	None	5169±2820 (3)	1239±49 (3)	727±108 (3)
Adult	Partial hepatectomy 3–8 days previously	—	1046±88 (10)	—

* Each organ pooled before enzyme assay.

DISCUSSION

Our own results with menthol confirm Fishman's (1940) findings in so far as the enzyme in liver and kidney is concerned. It seems, however, that Fishman suppressed repair processes by overdosage with menthol, and thus obtained a rise in glucuronidase which was only a small fraction of that provoked by the first doses of the compound. No explanation can be offered for our failure to observe the rise in the activity of spleen glucuronidase in menthol-treated mice reported by Fishman, unless the discrepancy has its origin in the wide variation in its glucuronidase level normally shown by spleen. This variation one might expect if glucuronidase activity is a measure of the amount of cell proliferation in progress.

The present work shows that the effects of menthol on β-glucuronidase activity *in vivo* bear no relation to its glucuronidogenic property, but are secondary to its hitherto unsuspected toxic action on liver and kidney. It seems impossible that chloroform, carbon tetrachloride, mercuric nitrate, phosphorus or uranyl acetate should give rise to a glucuronide in the body, and yet all these substances have been found to cause striking changes in glucuronidase activity. Of other substances which caused a rise in glucuronidase, phenylarsenoxide could conceivably form a derivative conjugated with glucuronic acid, and evidence has been obtained that sulphathiazole is partially excreted in rabbits as the glucuronide of a hydroxy derivative (Thorpe & Williams, 1940). In spite of the very large dose injected, sulphathiazole caused only a relatively small rise in glucuronidase activity in mice, and this was confined to liver. No change in glucuronidase was observed after injection of two compounds which are known to be glucuronidogenic, pregnanediol (Venning & Browne, 1936) and sodium sulphapyridine (Scudi, 1944). It should be pointed out that a change in experimental conditions might reveal an effect of sulphapyridine on glucuronidase in liver, since it produced some damage in this organ. The effect of menthol glucuronide on liver glucuronidase was due presumably to menthol liberated by the enzyme initially present. Fishman's theory (see p. 462) provides no explanation for a change in the enzyme brought about by administration of a compound already conjugated with glucuronic acid.

On the basis of the experiments described above it is not possible to decide whether β-glucuronidase is actually concerned in cell proliferation, or whether the increases in activity observed merely reflect an increase in metabolic activity. It is interesting to note, however, that the rise in the enzyme level occasionally slightly preceded the first appearance of cell division which was definitely in excess of normal. Whatever the cause of the parallelism between the glucuronidase activity in an organ and the amount of tissue growth in progress, it provides a straightforward explanation of the changes in the enzyme in liver and kidney which follow administration of menthol and other substances to mice. It seems possible that the same explanation can be applied to the effect of oestrogens on uterine glucuronidase (Fishman & Fishman, 1944; Fishman, 1947), and to a recent observation (Fishman & Anlyan, 1947), which suggests that in some cases of human carcinoma the tumour contained more glucuronidase than the corresponding normal tissue. The possible bearing of our results with carbon tetrachloride on the finding (Pincus & Martin, 1940) that, in liver poisoning produced by this compound, the physiological activity of oestrone is enhanced is of interest.

It is no longer necessary to speculate on the probable role of glucuronidase in the body in order to explain the changes in activity produced by extrinsic agents. The citation by Fishman (1947) of the work of other authors in support of his contention that the enzyme acts synthetically, however, makes it necessary to consider their results from this angle. Florkin, Crismer, Duchateau & Houet (1942) obtained evidence for the condensation of glucuronic acid with borneol in the presence of β-glucuronidase, but the percentage conjugation was very small under extreme conditions, and they concluded: 'Quant à

savoir si cette synthèse enzymatique correspond au mécanisme réalisé *in vivo*, c'est évidemment une autre affaire.' In the work of Lipschitz & Bueding (1939) and Crépy (1946) on the formation of conjugated glucuronides by surviving liver slices, there is no suggestion that the enzyme concerned is β-glucuronidase. De Meio & Arnolt (1944), who studied conjugation of phenol by surviving tissue slices, found that glucuronic acid reversed the inhibition of this process produced by iodoacetate. They also found that feeding phenol and borneol to rats increased phenol conjugation by liver and kidney *in vitro*. Their results are difficult to interpret since it is known that phenol may be conjugated with either sulphuric or glucuronic acid. Even if De Meio & Arnolt are correct in thinking that, contrary to the views of Lipschitz & Bueding (1939), glucuronides are formed by direct condensation of the 'aglucurone' with free glucuronic acid, there is no reason to believe that β-glucuronidase is responsible. With regard to De Meio & Arnolt's second finding, there is, in view of our own work, no need to postulate adaptation by the enzyme or enzymes responsible for conjugation of phenol, since both phenol and borneol may have caused liver and kidney damage in their experiments. Results obtained by Bueding & Ladewig (1939) in studying the effect of chloroform poisoning in guinea pigs on glucuronide synthesis by liver slices are of interest in this connexion. Not only did the liver slices from the poisoned animals show the usual increase in glucuronide synthesis on addition of lactate, but they were apparently more active in forming borneol glucuronide than slices from normal animals. The latter aspect of their results is not touched upon by the authors. While there is thus some evidence to suggest that, following damage to an organ, there may be an increase in its ability to form conjugated glucuronides, it is at present impossible to say whether or not this is due to the rise in β-glucuronidase activity observed when repair is in progress, nor is it certain that the glucuronides are formed directly from free glucuronic acid.

SUMMARY

1. The effect of menthol administration to mice in increasing the β-glucuronidase activity of liver and kidney is due to its toxic action on these organs. The rise in enzyme activity is associated with an increase in cell proliferation following injury. Menthol had no effect on spleen glucuronidase.

2. Among other substances examined, the following caused changes in glucuronidase in liver or kidney in an analogous fashion to menthol: chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, phenylarsenoxide, uranyl acetate, menthol glucuronide and sulphathiazole. The effect of chloroform on kidney glucuronidase was confined to male mice.

3. Livers from adult mice after sub-total hepatectomy, and spleens, livers and kidneys from infant mice showed high glucuronidase activities.

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The Use of Radioactive Isotopes in Immunological Investigations

1. PRECIPITIN REACTIONS WITH OVOVITELLIN CONTAINING P^{32}

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Analysis of the specific precipitates obtained from antigen-antibody mixtures can help to throw light on many problems of immunology; for example, the quantitative relationship between antigen and antibody in the precipitin reaction, the behaviour of 'inhibitory' substances, and the relative avidities of closely related antigens. Total N determinations on the antigen and on the specific precipitates have been used to determine the antibody/antigen ratio (Heidelberger & Kendall, 1929; cf. Heidelberger, 1939), and this method is of particular value when polysaccharide antigens containing little or no N are used. Use has also been made of antigens containing readily determinable elements or groups which are absent from the antibody. Amongst such 'labelled' antigens are haemoglobin (Wu, Cheng & Li, 1929), iodoalbumin (Wu, Sah & Li, 1929), haemocyanin (Hooker & Boyd, 1936) and proteins conjugated with dyes (Heidelberger & Kendall, 1930, 1935). For a somewhat different purpose N^{15} has been used as a tracer for investigations on the formation and the circulation of antibodies (Schoenheimer, Heidelberger, Rittenberg & Ratner, 1941; Schoenheimer, Ratner, Rittenberg & Heidelberger, 1942; Heidelberger, Treffers, Schoenheimer, Ratner & Rittenberg, 1942).

In the present investigation an attempt has been made to use antigens containing radioactive tracer elements for serological investigations. These elements can be determined in extremely minute amounts, and the determinations are not affected by the presence of relatively large amounts of the corresponding non-radioactive element. Two convenient isotopes for this type of work are S^{35} and P^{32}.

For the preparation of radioactive antigens the following methods were considered: (i) conjugation of a natural protein with a simple organic substance containing the radioactive element; (ii) direct phosphorylation of natural proteins or of dephosphorized casein, using phosphoryl chloride prepared from radioactive P; (iii) a biological preparation of a protein containing the particular element whose radioisotope is to be used (e.g. vitellin containing P^{32}, haemoglobin containing Fe^{55} or Fe^{59}). With regard to the first method, Berenblum & Wormall (1939), and Boursnell, Francis & Wormall (1946) have investigated the serology of proteins treated with $\beta\beta'$-dichlorodiethylsulphone, and Banks, Boursnell, Francis, Hopwood & Wormall (1946) have described the preparation of mustard gas sulphone-protein derivatives containing radioactive sulphur (S^{35}). We have recently used this S^{35}-containing conjugated antigen, and phosphorylated proteins containing P^{32}, for some preliminary serological investigations, and a brief account of the results has been given elsewhere (Boursnell, Dewey, Francis & Wormall, 1947). The present paper deals with the preparation of hen ovovitellin containing P^{32} and its use in quantitative precipitin reactions.

METHODS

Collection of radioactive eggs. In the first experiment, two hens (nos. 815 and 816) were each given an intramuscular injection in the right thigh of 50 microcuries (μC) of P^{32} (in the form of neutral sodium phosphate, in 1–1·5 ml. of 0·9% (w/v) NaCl solution). In the second experiment, hen 816 was given a single injection of 380 μC, about 1 month after the first. The eggs were collected for 1 month after the first injection and worked up in small batches (1 or 2 eggs laid on the same day, for the first 12 days, and batches of 7 eggs thereafter). In the second experiment a vitellin preparation of relatively high radioactivity was obtained from the eggs laid on the 4th, 5th and 6th days following the injection.

Separation of vitellin. This was carried out essentially by the method of Calvery & White (1932). The separated and washed yolks were punctured, and the membrane contents allowed to flow out into 20 ml. of 10% (w/v) NaCl per yolk. As much lipid as possible was removed by extraction with ether containing 2% ethanol (6–8 extractions), and the vitellin in the saline solution was then precipitated twice with 20 vol. of tap water. (It was found that some samples of ether caused denaturation of the vitellin, rendering it insoluble in 10% NaCl, but freshly opened bottles of ether, sp.gr. 0·720 (British Drug Houses Ltd.), were satisfactory.) For most purposes the final product was dissolved in 10% NaCl to give a total vol. of 50 ml. per egg, and used in this form ('soluble' or lipovitellin).

In a few experiments, however, further removal of lipid was effected, either by the hot ethanol treatment of Calvery & White (1932) or by pouring the cooled NaCl solution into 5 vol. of a cooled 7 : 3 ethanol-ether mixture, centrifuging, washing twice with ethanol-ether and twice with water, all these operations being carried out at 0° to −5°. Both extraction processes gave products ('insoluble' vitellin) with approximately the same P/N ratio (about one half that of the lipovitellin), but both rendered the products

insoluble in 10% NaCl. The product obtained by the cold ethanol-ether method was, however, soluble in saturated urea solution.

Preparation of radio-lecithin. The total ether extracts of the radioactive yolks were dried over anhydrous Na_2SO_4, evaporated to dryness, and the residues redissolved in dry ether. The radio-lecithin was separated from this solution by pouring into acetone and purified by redissolving in chloroform and reprecipitating with acetone. The final product was dried *in vacuo* over $CaCl_2$. In some serological tests radio-lecithin was added to an antiserum; this was effected by emulsifying the lecithin with normal rabbit serum, centrifuging and adding the supernatant fluid to the antiserum.

Radioactivity of the eggs. The main object of these experiments was the preparation of radio-vitellin, and at a later stage radio-lecithin, for immunological experiments, but a few measurements on the individual eggs, and on certain fractions of each egg, were made in order to obtain some idea of the rate of elimination of the injected P^{32} in various fractions of the egg. Hevesy & Hahn (1938) and Aten (1939) have studied the radioactivity of the lecithin fraction of the eggs of hens injected with P^{32}, but no specific information about the vitellin was available to us when our investigations were started. Radio-determinations were made on (*a*) the whole intact egg, (*b*) the whole yolk, (*c*) the separated total lipid (i.e. the ethanol-ether soluble fraction of the yolk) and (*d*) the separated lipovitellin. The values recorded for (*a*) and (*b*) do not, of course, represent the total radioactivity of the material concerned, but they serve as a rough comparison of the P^{32} content of the shells and yolks of different eggs and they give some idea of the rate of elimination of the injected P^{32} in these two fractions of the egg.

For the intact egg 'count', the egg was placed directly under the window of the Geiger-Müller counter. After the egg had been broken, the empty shell was, in some cases, roughly reconstituted into its original shape, and was found to have almost the same radioactivity as the original egg; calculations showed that the white of the egg had absorbed over 75% of the radiations from the yolk. The radioactivity of the white was usually so small that it contributed little to the activity of the intact egg. Thus the 'counts' for the intact egg are of value in giving an approximate relative value for the radioactivity of the shell.

The whole washed yolk (and in some cases the whole white) from each egg was placed separately in small glass crystallizing dishes and 'counted'. These values also are merely relative, and they should not be compared with the values for the intact egg or the more accurate figures for the separated lipids and lipovitellin; they merely serve to give an idea of the day-to-day variation in the radioactivity.

For the yolk lipid 'counts', samples of the total ethanol-ether extract from each batch, corresponding to 1/10th of an egg, were evaporated in 20 ml. porcelain crucibles which were then placed under the counter. Similar determinations were made on fractions (each equivalent to 1/50th of an egg) of the final NaCl solution of lipovitellin from each batch, the solution being evaporated on a brass sample plate, and radioactivity determinations made. Tests showed that the amounts of material present in the samples were not sufficient to lead to any significant loss of the P^{32} radiation by 'self-absorption', and since the dimensions, and geometrical arrangements relative to the counter windows, of the lipid and vitellin layers were approximately the same, a general comparison of the two sets of results seems justifiable.

Immunization. Rabbits were injected with non-radioactive vitellin, two rabbits being immunized with the 'soluble' and three with the 'insoluble'. In each case intraperitoneal injections of 5 ml. of a solution (or suspension) in 10% NaCl were given, containing about 3% of protein and 0·25% phenol (as a preservative), at intervals of 7–10 days. Precipitin tests were carried out on sera taken from the rabbits 7–10 days after the fourth and subsequent injections. Good antisera were obtained from both rabbits injected with 'soluble' vitellin and one injected with 'insoluble' vitellin; the other rabbits yielded weak antisera.

Precipitin tests. Antiserum diluted with an equal vol. of 20% NaCl (0·4 ml.) was mixed with 2·0 ml. of diluted antigen in a 15 ml. tapered centrifuge tube. (The antigen was previously centrifuged for 20 min., and dilutions were made with 10% NaCl to avoid precipitation of the vitellin by a reduction in the salt content.) After 3 hr. at 37°, with occasional stirring, the tubes were centrifuged for 20 min. and the supernatant fluids discarded. The inside of each tube was washed down with 6 drops of 10% NaCl, care being taken to disturb the precipitate as little as possible, and the tubes were centrifuged for a further 7 min. The washed precipitates were dissolved in 4 drops of water plus 4 drops of 0·5N-NaOH, and the solutions transferred to brass sample plates (containing kaolin). Each tube was washed out three times with 3 drops of water each time, and the washings added to the sample plates, which were then placed on a warm water bath until dry. A control experiment, with exactly the same technique, was carried out with normal rabbit serum added to the antigen solutions. Any radioactivity observed in the control, which was appreciable only with high concentrations of antigen, was subtracted from the value obtained for the corresponding mixture of antigen and antiserum.

Total P *determinations.* These were made by King's (1932) modification of the Fiske & Subbarow (1925) method.

Radioactivity measurements. These were made with the Geiger-Müller counter system described by Banks (1946). Owing to the much greater penetrating power of the radiations from P^{32} as compared with those from S^{35}, however, it was not found necessary to incinerate the materials before they were 'counted'. The plates were of the usual pattern, but were fitted with disks of 2 cm. diameter to improve the geometrical arrangements of the sample relative to the counter window. To facilitate the spreading and drying, about 50 mg. of kaolin were first suspended in a few drops of 50% ethanol and this suspension evaporated on the plate. The solution to be 'counted' (up to 0·5 ml.) was dropped on the kaolin pad and the plate heated over a warm water bath; the dried sample was protected against damage by adding 10–15 drops of 1 : 50 collodion in ethanol-ether mixture and was then redried.

This simple evaporation method for the preparation of samples for P^{32} determinations on serological precipitates and other organic material containing the radio-isotope greatly facilitates the determinations. It is desirable that, where this method is used, checks be made by incinerating some of the material and separating the phosphate as magnesium ammonium phosphate for radioactivity determinations. This control procedure has been adopted in the work described in this paper and in all cases the simple 'evaporation' method has given essentially the same result as the digestion or incineration method.

RESULTS

The excretion of P^{32} in various fractions of the eggs laid by hens injected with P^{32}-containing phosphate

The total radioactivities of the shell, whole yolks, and lipid and lipovitellin fractions of the eggs laid by the two hens during the month following the injection of 50 μC of P^{32} into each hen are given in Figs. 1 and 2. Owing to normal variations between individual eggs, particularly those laid by different hens, the points do not always lie on a smooth curve; but it is quite evident that the radioactivity of the vitellin and lipid fractions of the yolks reached a maximum about the 4th day after the injection, remaining approximately constant for a further 6 or 7 days, and then decreasing rapidly. For most of our subsequent immunological work, eggs laid 4–11 days after the injection of P^{32} have been used for the preparation of radio-vitellin and radio-lecithin. At the maximum, each egg contained in the lipovitellin plus lipid about 1% of the total P^{32} injected, with about two thirds of this in the lipid fraction. The activities given for the shells and the whole yolks are, as pointed out above, purely arbitrary values and have only relative significance.

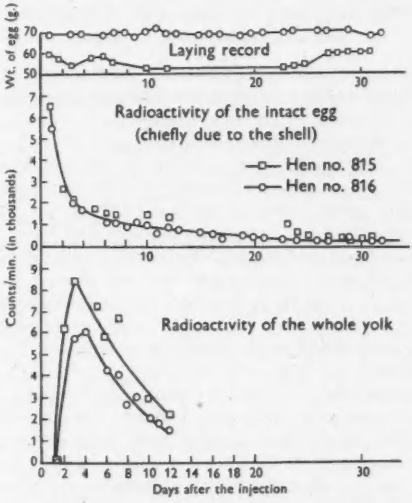

Fig. 1. Radioactivity of the intact egg (i.e. shell) and the yolk of eggs from hens injected with phosphate containing P^{32}. The values given in these figures are not absolute values, but merely give the relative activities of the shell (or yolk) from day to day (see text).

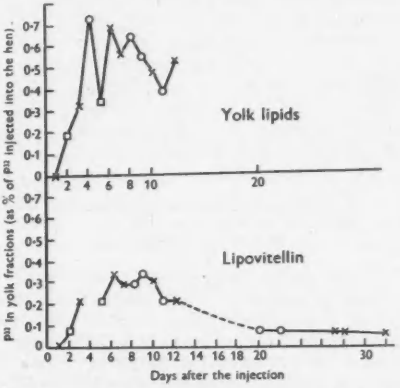

Fig. 2. Radioactivity of the yolk lipids and lipovitellin of eggs from hens injected with phosphate containing P^{32}. The values are expressed as activity/single egg. From the 1st to 12th day, each individual egg was examined; thereafter the eggs were grouped in batches of seven. Hen no. 815 —□—. Hen no. 816 — ○—. Mixed yolks (815 + 816) —×—.

The determination of P^{32} in vitellin-anti-vitellin precipitates

Table 1 gives the results of determinations of the amount of antigen in the precipitates obtained by treating 0·20 ml. samples of a lipovitellin antiserum

Table 1. *Radio-P content of precipitates with 'radio'-vitellin and rabbit anti-vitellin serum*

(0·20 ml. of antiserum + 0·20 ml. of 20% (w/v) NaCl were added to 2·0 ml. of 'soluble' radio-vitellin in 10% (w/v) NaCl. Precipitates were centrifuged after 3 hr. at 37°. Control: normal rabbit serum instead of antiserum.)

Concentration of antigen (% protein)	Radio-P in 2 ml. antigen (μg.)	Radio-P precipitated: Rabbit antiserum* (μg.)	Control serum (μg.)	Average minus control (μg.)	As % of total antigen P
2·3	942	9·45, 12·4	13·4	0·0	0·0
0·37	157	30·9, 32·6	0·6	31·1	20
0·13	52·3	18·3, 18·3	0·5	17·8	34
0·042	17·4	6·34, 7·02	0·4	6·3	36
0·014	5·81	2·2, 2·1	0·0	2·1	37
0·0046	1·94	0·85, 0·61	0·0	0·7	37

* Duplicate experiments.

(no. 457) with 2·0 ml. samples of solutions containing radioactive lipovitellin. (Both lipovitellin and 'insoluble' vitellin injections produced antisera capable of precipitating lipovitellin.) It can be seen that, with the lower concentrations of antigen, approximately the same fraction (about 37 %) of the total P^{32} present in the antigen solution was precipitated in each case, down to extremely low concentrations of antigen. With moderate excess of antigen, the percentage of antigen P precipitated was less, while with a large excess none of the P^{32} was precipitated, thus confirming the visual observation in the precipitin test mixtures.

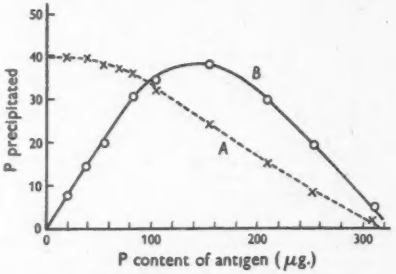

Fig. 3. P^{32} content of lipovitellin-antilipovitellin precipitates. *A*, percentage of total antigen P precipitated. *B*, total P precipitated (μg.). Antigen: 2·0 ml. radio-lipovitellin (P/N ratio = 0·14) in 10 % NaCl. Antiserum: 0·2 ml. rabbit antiserum to lipovitellin + 0·2 ml. 20 % NaCl. Precipitates: centrifuged after 3 hr. at 37° and washed once with 10 % NaCl.

In a further experiment a much smaller range of antigen concentration was covered, involving the use of antigen (radio-lipovitellin) solutions varying in strength from 0·05 to 0·74 %. This range covers the points of maximum precipitation and extends into the excess-antigen inhibition zone, under the conditions of our experiments. From the results of this experiment (Fig. 3) it will be seen that, with the lowest concentrations of antigen, there was again precipitation of about 40 % of the antigen P. Where there was the maximum amount of P^{32} in the precipitate, this represented about 25 % of the P^{32} in the antigen solution used.

The part played by lipids in the lipovitellin-antivitellin precipitin reaction

Since vitellin preparations, particularly the 'soluble' variety, contain considerable amounts of phospholipins, a few experiments have been made to determine whether these take part in the precipitin reaction. Varying amounts of radio-lipovitellin were added to measured volumes of anti-vitellin serum, four tubes being set up for each antigen dilution. After the mixture had been incubated and centrifuged, two tubes of each mixture were extracted three times with an ethanol-ether mixture to remove lipid matter. P^{32} determinations were made on the unextracted and the extracted precipitates and on the ethanol-ether extracts. These results (Table 2) showed that 70–80 % of the P^{32} in these specific precipitates was of phospholipid type (i.e. extractable with ethanol-ether). Since the lipovitellin preparations *A* and *B* used as antigens contained only 47 and 41 % respectively of their total P^{32} as phospholipin, there appears to have been preferential precipitation of much of the phosphatide by the antiserum. Parallel control tests made with an unrelated precipitin reaction (human serum plus anti-human serum) carried out in the presence of radio-lipovitellin gave precipitates containing no significant amount of P^{32}. It is concluded, therefore, that the preferential precipitation of phospholipin in the lipovitellin-antivitellin precipitates is not simply due to the mechanical carrying down of lipid by the precipitate.

Table 2. *The lipid* P *of lipovitellin-antivitellin precipitates*

(Precipitin reactions as in Table 1. Some of the precipitates were extracted three times at room temperature with 5 ml. of 7 : 3 ethanol-ether for 30 min. each time. The extracted precipitates were dissolved in dilute NaOH, and the ethanol-ether extracts were concentrated to small bulk. Both solutions were then transferred to sample plates for P^{32} determinations.)

		Radio-P content			
			Lipovitellin-antivitellin precipitate		
				Extractable by ethanol-ether	
Exp.	Concentration of antigen (%)	Antigen* (μg./2 ml.)	Total (μg.)	(μg.)	As % of precipitated P
1	0·377	157·5	37·7	26·9	71
	0·251	104·7	34·0	24·7	73
2	0·322	152·4	7·4	5·8	78
	0·214	101·6	11·3	9·0	80
	0·161	76·2	10·2	7·9	78

* 47 % of the P^{32} of the lipovitellin (preparation *A*) used as antigen in Exp. 1 was extractable with ethanol-ether, and the corresponding value for preparation *B* (Exp. 2) was 41 %. *A* and *B* had P/N ratios of 0·14 and 0·158, respectively.

In other experiments radio-ovolecithin has been added to mixtures of non-radio-lipovitellin and antivitellin. The specific precipitates formed in these mixtures contained very appreciable amounts of radio P. Further experiments are being made to determine whether this is simply due to interchange of lecithin between the solution and the antigen, or whether it is due to the direct combination of the radio-lecithin with the antivitellin or with the antigen-antibody complexes.

DISCUSSION

For the preparation of a 'labelled' protein which can be used as an antigen, the injection of P^{32} into hens and the separation of P^{32}-containing vitellin from the eggs laid by these hens has proved very satisfactory, with a reasonably good utilization of P^{32}. The injection of P^{32} is known to lead to the early appearance of the radio isotope in the various P-containing compounds of the hen and its eggs (Hevesy & Hahn, 1938; Aten, 1939; Chargaff, 1942*b*). In our experiments, the shell of the egg laid the day after the injection was highly radioactive, but that laid on the second day had only about half this activity; subsequently, the radioactivity of the shells fell to a relatively low level (Fig. 1). The whites of the eggs showed very little radioactivity, particularly after the first day or two, but the P^{32} content of the yolk lipids and the lipovitellin increased fairly rapidly up to a maximum at about 4 days after the injection, and then decreased slowly (Fig. 2).

These results indicate that the eggs laid 4–11 days after the injection of P^{32} into the hen serve best as sources of P^{32}-containing vitellin and lecithin. In one experiment, for example, about 2% of the injected P^{32} was present in the lipovitellin and a little over 4% in the yolk-lipids of the eggs laid during this period. Labelled vitellin and lecithin can thus be readily obtained with a specific radioactivity adequate for many immunological purposes, including the use of these compounds in micro-precipitin tests and for some *in vivo* immunity experiments. In many of the precipitin tests described in this paper, rather large amounts of antigen solution were used, because of the relatively small amount of P^{32} available to us, but it is possible, by injecting more P^{32} and by adopting a special micro-precipitin-test technique, to make reliable P^{32} determinations on precipitates from considerably less than 0·10 ml. of antigen. Although we used relatively large volumes of antigen solution for the tests, it should be remembered that some of our precipitates which gave 'counts' which could be measured accurately, were obtained from less than 0·05 ml. of the stock antigen solution (2·3% radio-lipovitellin in 10% NaCl solution).

Lipovitellin is not an ideal protein complex for serological investigations, partly because of its relative insolubility and partly because it contains a very considerable amount of bound phosphatide. The first difficulty can be overcome by adding NaCl, to give a concentration of 5–10% (w/v), to all the solutions used for the precipitin tests; but the second problem cannot be solved so easily. The extraction of the firmly bound phosphatide with hot ethanol or with ethanol-ether mixtures in the cold renders the vitellin almost insoluble in water or salt solutions. It is then practically useless for precipitin tests, although it might be satisfactory for complement-fixation tests.

The immunological properties of vitellin have been studied by several authors. Wells (1909, 1911) showed, by anaphylactic experiments, that it is antigenic and that it is serologically distinguishable from ovomucoid and ovalbumin. Emmerich (1913) and Seng (1913) investigated the serological cross reactions between the yolk proteins of the hen's egg and those of the eggs of other birds, but as no separation of the vitellin was carried out, many of their results might well have been due to the other proteins present; hen egg-yolk livetin, for example, is antigenic, and immunological tests show that it is closely related to hen serum globulin (Jukes & Kay, 1932). Roepke & Bushnell (1936), using 'soluble' vitellin, obtained antisera which gave specific precipitation, and they were able to show that the serum of laying hens contains significant amounts of a protein similar to vitellin.

In our experiments immunization was effected with both the 'soluble' and the 'insoluble' vitellin, and both preparations produced good antisera in rabbits: since both these antisera gave precipitates with 'soluble' vitellin it is concluded that at least some of the reactive groupings in the antibodies are directed against groups of the protein molecule. Whether or not these antibodies can react with the lipid groups of lipovitellin is not known. The yolk phosphatides have been carefully studied in more recent years by Chargaff (1942 *a*, *b*; cf. also Chargaff, Ziff & Rittenberg, 1942) who found that removal of all the uncombined lipids with ether and repeated dialysis yielded a lipovitellin containing about 18% of phosphatide and a total P content of 1·5% (compared with 1·0% in the ethanol-extracted product). Our 'soluble' vitellin preparations contained about the same percentage of phosphatide (15–22%) as did the preparations of Chargaff, accounting for about half the P of the 'soluble' vitellin.

The specific precipitates obtained in our experiments contained considerable amounts of phosphatide, more in fact than did the lipovitellin used as antigen. In one experiment (e.g. Exp. 2, Table 2) about 80% of the P^{32} of the precipitates was due to phospholipin, as against a figure of 41% for the antigen. The combination (or perhaps adsorption) of the phosphatide by the antigen-antibody complex

appears to be specific since a heavy precipitate formed between human serum proteins and their antibodies in the presence of radio-lipovitellin did not carry down any significant amount of phospholipin. It seems possible, therefore, that the lipovitellin antibodies have groups capable of reacting specifically with ovophosphatide. Precipitation might only occur in these mixtures when the antibodies have also reacted with the protein vitellin or with lipovitellin. It is however difficult to explain how the antibodies detach phosphatide from a lipoprotein complex, from which the phosphatide cannot be removed by ether extraction.

The nature of the linkage between ovophosphatide and the protein vitellin is not at all clear, and it would be of interest to know whether there is any significant serological difference between vitellin and lipovitellin. Removal of the ether-soluble substances from serum does not affect the true antigenic properties of the serum, i.e. its power to produce antibodies on injection, but specific precipitation does not occur when both antigen and antiserum are fully extracted with cold ethanol-ether (Hartley, 1925). Other specific precipitates vary in their lipid content, for whereas Horsfall & Goodner (1936) found 4–51 % of lipid in their pneumococcus polysaccharide precipitates, Marrack & Smith (1931) were unable to find any lipid in horse serum pseudoglobulin antigen-antibody precipitates.

The experiments described in the present paper were designed to indicate how a radio-isotopic tracer might be of value in one branch of immunochemistry. The results show that the tracer can be determined quite readily in precipitates obtained in ordinary precipitin tests. In these experiments no attempt was made to get maximum precipitation in all cases, e.g. by keeping the antigen-antibody mixtures in the refrigerator for several days, nor were any special efforts made in most of these experiments to purify the vitellin used as antigen, for it was not our desire to remove the firmly bound lipid from the vitellin. The results of other experiments, with fully extracted lipid-free vitellin, will be reported later.

SUMMARY

1. P^{32}-containing vitellin prepared from eggs laid by hens injected with P^{32}-containing phosphate has been used for immunological investigations.

2. P^{32} determinations have been made on precipitates obtained in ordinary precipitin tests to determine the amount of antigen in vitellin-antivitellin precipitates.

3. This isotopic tracer method, together with total-N determinations, will readily allow antigen/antibody ratios to be determined. Other possible uses of radio-isotopes for the study of immunochemistry are indicated.

4. Lipovitellin-antivitellin precipitates contained a much higher ratio of phosphatide P^{32}/protein P^{32} than did the lipovitellin antigens. This preferential carrying down of phosphatide by the precipitate is specific, and it does not occur in precipitin reactions unrelated to vitellin, e.g. in the precipitation of human serum proteins by their antibodies.

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N^1-Diethylsulphanilamide: a Reagent for the Colorimetric Estimation of Thyroxine

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The estimation of thyroxine in thyroid substance can be performed by biological assay, or by the determination of 'thyroxine iodine' in the hydrolyzed material. In the latter case the iodine content of the precipitate at pH 4·5, or of its butanol extract after treatment with a strongly alkaline aqueous phase, which retains the diiodotyrosine, can be expressed as 'thyroxine'. The methods have been reviewed by Harington (1933) and Salter (1940). On adapting the original butanol-extraction procedure of Leland & Foster (1932), as modified by Blau (1935), to artificially iodinated proteins, Reineke, Turner, Kohler, Hoover & Beezley (1945) compared their results with those of biological assay in the guinea pig and were led to conclude that the chemical method reliably accounted for the thyroxine present; the properties of any non-thyroxine component of the butanol layer would need to parallel closely those of thyroxine itself. From an examination of thyroid-gland preparations from different species, Parkes (1946) concluded that a fairly close relationship exists between acid-insoluble iodine (and total iodine) and the biological activity as determined in the *Xenopus* tadpole. On the other hand, Deanesly & Parkes (1945) found a very marked divergence from this correlation in the case of the acid-insoluble-iodine fractions from artificially iodinated proteins.

It would seem desirable to have an alternative method for the chemical assay of thyroxine, and we have been engaged in investigating the possibility of developing a specific colour reaction which could be quantitatively applied. Thyroxine gives a characteristic, though non-specific, red colour with nitrous acid, under special conditions, while the ninhydrin test is weakly positive (Harington, 1933). Recently Roche & Michel (1946) have reported a method for the quantitative estimation of thyroxine and diiodotyrosine based on the nitrous acid reaction. A further qualitative test was described by Komant (1930) in which a red or purple colour, transient in dilute solution, was produced by thyroxine in the presence of diazotized sulphanilic acid in alkaline solution. A colour, though much less intense, was also given by diiodotyrosine. Since both the 3′ and 5′ positions in thyroxine are occupied, the reaction mechanism, as inferred but not proved by Komant, presumably involves the replacement of the iodine at one of these points by the arylazo grouping. Thyroxine *O*-methyl ether does not react under these conditions (Loeser, Ruland & Trikojus, 1938). In examining this reaction further we have selected as reagent N^1-diethylsulphanilamide from a number of diazotizable amines using sensitivity, stability of the colour formed and the preference of the reaction product for the butanol phase in *n*-butanol-water mixtures as criteria. This last property was taken into account since, by transferring to butanol, thyroxine may be conveniently separated from contaminants, such as diiodotyrosine, prior to assay. The conditions of the reaction have also been studied with the object of increasing the thyroxine specificity and in order to standardize a procedure suitable for quantitative analysis.

EXPERIMENTAL

Preliminary studies

Selection of amine

Sulphanilic acid was chosen by Komant (1930) rather than its arsenic analogue, *p*-nitraniline or *p*-aminobenzoic acid, all of which gave, after diazotization, coloured reaction products with thyroxine in an alkaline medium (optimum pH 9–10). In Table 1 is listed a number of amines together with the results of our attempts to couple them with thyroxine, at pH 9–10, the test solutions being constituted as follows:

Thyroxine. A 0·1 % (w/v) solution in 0·004 N-NaOH.

Alkaline solution. NaOH (20 g.) and $NaHCO_3$ (28·8 g.) were dissolved in water and diluted to 1 l.

Diazo solution. In most cases the direct method of diazotization was used. The test amine (0·005 mol.) was dissolved or suspended in 9 ml. of 10 N-HCl, water was added to a volume of 100 ml. and 5 ml. of this solution or suspension was mixed with 25 ml. of 4·5 % (w/v) aqueous $NaNO_2$ at 0° and allowed to stand for 10 min. Diazotization was checked by coupling a sample of the solution with α- or β-naphthol under conditions comparable to those used for the reaction with thyroxine. Only those amines whose diazotized solutions gave a strong positive reaction with

Table 1. *Reactions between thyroxine and various diazotized amines in aqueous solution* (pH 9–10) *and the solubilities, relative to the aqueous phase, of the coloured products in* n-*butanol*

No.	Diazotized amine	Colour: Thyroxine (0·1 %)	Colour: Control (no thyroxine)	Transferred to *n*-butanol phase
1	Sulphanilic acid	Deep red	Yellow	–
2	Sulphanilamide	Red	Yellow	+
3	N^1-Ethylsulphanilamide	Rose-red	Pale yellow	+
4	N^1-Diethylsulphanilamide	Rose-red	Pale yellow	+
5	Sulphaguanidine	Red	Yellow	+
6	*m*-Sulphaguanidine	Red	Deep yellow	Partly
7	Sulphapyridine	Orange	Yellow	+
8	Sulphadiazine	Deep red	Yellow	–
9	Sulphathiazole	Red	Orange-yellow	–
10	Sulphamerazine	Yellow-red	Orange-yellow	–
11	1-Aminonaphthalene-4-sulphonamide	Brownish yellow	Brownish yellow	–
12	1-Aminonaphthalene-4-sulphonic acid	Brownish yellow	Brownish yellow	–
13	2-Aminonaphthalene-4:8-disulphonic acid	Red	Orange-yellow	–
14	4-Aminobenzoic acid	Red	Yellow	–
15	Ethyl 4-aminobenzoate	Red	Yellow	+
16	3:5-Dibromo-2-aminobenzoic acid	Yellow	Yellow	–
17	4-Aminoacetophenone	Brown-red	Yellow	+
18	*p*-Nitraniline	Red (purplish)	Deep yellow	+
19	2:4-Dichloroaniline	Red	Pale yellow	+
20	2:5-Dichloroaniline	Deep pink	Pale yellow	+
21	4-Diethylaminoaniline	Yellow	Yellow	–
22	*o*-Toluidine	Yellow	Yellow	–
23	*m*-Toluidine	Deep orange	Yellow	+
24	*p*-Toluidine	Orange	Yellow	+
25	2-*p*-Xylidine	Yellow	Yellow	–
26	4-*m*-Xylidine	Yellow	Yellow	–
27	*p*-Anisidine	Yellow	Yellow	–
28	4-Aminodiphenyl ether	Brownish yellow	Brownish yellow	–
29	4-Aminodiphenylamine	Brownish yellow	Brownish yellow	–

either of these two phenols are included in the table. The large excess of nitrite is as used by Komant (in our later studies with N^1-diethylsulphanilamide this reagent was appreciably reduced in quantity without affecting the reaction).

Procedure. The thyroxine solution (1 ml.) was mixed with the alkaline solution (1 ml.) and the solution of the diazotized amine (1 ml.) then added. A colour, when produced, was formed almost immediately. Redistilled *n*-butanol (1 ml.) was added after 3 min. and the tube was well shaken. A control (1 ml. of 0·004 N-NaOH) was treated in the same way.

Comments on Table 1. Although a systematic study of the relationship between amine structure and reactivity with thyroxine was not made, the presence of certain substituents in the position *para* to the amino group precluded reaction with the amino-acid but not with naphthol under the conditions used. If the colours in the control tube and in that containing thyroxine were comparable a negative reaction was recorded. This occurred when the *p*-substituent was $—N(CH_3)_2$, $—OCH_3$, $—OC_6H_5$ and $—CH_3$. Unless the substituent is halogen (amines 19, 20) it would seem that both positions *ortho* to the amino group should be unoccupied (amines 22, 25, 26). This may explain the apparent anomalies of negative reactions with the naphthalene analogues of sulphanilic acid and its amide (amines 11, 12). With the amino group in the 2-position in naphthalene (amine 13) the reaction was strongly positive.

Derivatives of aniline substituted in the *para* position by $—NO_2$, $—COCH_3$, —COOH, $—COOC_2H_5$, $—SO_3H$, $—SO_2NH_2$, $—SO_2NHR$, and $—SO_2NRR'$ all gave a positive reaction although not all of the pigments formed were of equal intensity of colour, and they varied in their degree of preference for the aqueous or *n*-butanol phase. The group of sulpha drugs and 2:4-dichloroaniline gave the best results. N^1-Diethylsulphanilamide was made the subject of further study owing to the stability of its diazonium salt, the readiness with which the pigment transferred to the *n*-butanol phase and the light colour of the control. This amine was also more easily diazotized than 2:4-dichloroaniline and had the further advantage of freedom from halogen. There is, of course, the possibility that other amines may prove even more suitable, particularly since, in our subsequent studies with N^1-diethylsulphanilamide, the conditions for diazotization and colour development eventually adopted differed materially from those used in the above comparative tests.

Observations on the conditions of Komant's test

Komant described two sets of conditions leading to the formation of coloured products between thyroxine and diazotized sulphanilic acid: (*a*) in which equal volumes (1 ml.) of solutions of thyroxine (0·1 %), the diazonium chloride and Na_2CO_3 (10 %) were mixed to give a red colour (the colour tone changing gradually to brown although in more dilute solutions of thyroxine the colour was found to fade in a few minutes), and (*b*) in which 5 vol. of the diazonium chloride solution were added. Under the latter conditions Komant observed the production of a violet colour which rapidly faded on passing a current of oxygen through the solution. Qualitative observations have shown that these subsequent changes of colour are largely dependent upon pH. Thus, if under conditions (*a*) (pH 9–10) 2N-NaOH (1 ml.) is added immediately after colour development, the initial colour quality is retained for several days, even with dilute (0·005 %) solutions of thyroxine, while that of the control (no thyroxine) is straw rather than dark yellow. Similarly, under conditions (*b*) (pH about 8·5) the initial red-purple colour does not change to violet and then to brown, but persists for several days if alkali (1 ml. 10N-NaOH) is immediately added. Moreover, if NaOH is not added, the subsequent decompositions appear to be independent of reactions with oxygen, since in controlled tests the bubbling of oxygen, air or nitrogen effected the changes (without, however, the development of an intermediate violet coloration) at similar rates; even mechanical agitation of the solution in a closed tube caused the initial red-purple colour to pass rapidly to reddish orange, whereas that of the contents of a similar stationary tube changed gradually *through violet* to brown. The colour changes are probably mainly due to side reactions of diazotized sulphanilic acid, these being inhibited at high pH.

Investigations with N^1-diethylsulphanilamide. Effects of pH and other factors on the production, stability and specificity of the colour

Reaction at pH 11–12. Under the conditions used for the comparative test (pH 9–10, Table 1) diazotized N^1-diethylsulphanilamide also produced a colour with diiodotyrosine (orange red) and tyrosine (deep yellow) while the control was yellow. Although diiodotyrosine can be separated from thyroxine by partitioning between aqueous NaOH and *n*-butanol, tyrosine is more evenly distributed between the two phases. A differentiation was made possible by carrying out the reaction at a higher pH as described below. It may be noted that colour development also depends upon the order of mixing of the reactant solutions, thyroxine, diazonium salt and alkali and *n*-butanol and that the addition of a further amount of alkali both intensifies and stabilizes the colour initially produced (as mentioned above for the colour formed with diazotized sulphanilic acid).

Thyroxine solution (0·004 %; 1 ml.) was mixed with *n*-butanol (1 ml.) and then 1 ml. of aqueous NaOH of selected concentration was run in. The mixture was shaken and the solution of the diazonium chloride (1 ml.) was then added and shaking continued for 30 sec. 2N-NaOH (1 ml.) was then immediately added. The results of these tests and of those carried out at the same time with diiodothyronine, diiodotyrosine, tyrosine and control solutions are given in Table 2. It can be seen that the range 0·2–0·3N-NaOH provides optimum conditions (pH 11–12) for the production of an immediate colour of good intensity with a pale control and negative results with tyrosine and diiodotyrosine. The alkali initially added, if below 0·15N, produced only yellow or brown colours even after 24 hr., whereas if above 0·5N, a colour developed only on standing. In order to check the optimum pH for the development of an immediate colour, a series of tests was carried out with buffer solutions over the pH range 10–12·8. The buffer was formed from glycine, NaOH and NaCl (Walbaum, 1920). The solution of thyroxine (0·1 %; 1 ml.) in buffer at the test pH was shaken with butanol (1 ml.), and 1 ml. of the diazo solution (previously brought to pH 7) was added. The results confirmed the pH range 11–12 as optimal; the conditions of the buffer medium were not, however, suitable for general adoption, as the control was yellow (due to glycine) with a corresponding yellow tint in the test colour.

Table 2. *Colours developed between diazotized N^1-diethylsulphanilamide and thyroxine and related compounds at varying* pH (*strength of alkali added as indicated*). *Reaction in water—*n*-butanol. 30 sec. after mixing the reagents additional alkali* (2N-NaOH; 1 *ml.*) *was added to the tubes and the colours of the butanol phases noted; after standing for* 24 *hr. the colours were again observed*

NaOH (1 ml.) ...	0·05N		0·1N		0·2N		0·3N		0·5N		N	
Time ...	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.	30 sec.	24 hr.
Thyroxine (0·004 %; 1 ml.)	Yellow	Orange	Brown	Brown	Light purple	Light purple	Deep pink	Light purple	Pale pink	Deep pink	No colour	Pink
3:5-Diiodothyronine (0·004 %; 1 ml.)	Yellow	Brown	Brownish pink	Orange brown	Pink	Pink	Pale pink	Light pink	Straw	Pale pink	No colour	Straw
3:5-Diiodotyrosine (0·004 %; 1 ml.)	Yellow	Yellow	Deep yellow	Yellow	Straw	Light yellow	No colour	Straw	No colour	Straw	No colour	Straw
Tyrosine (0·004 %; 1 ml.)	Light yellow	Yellow	Deep yellow	Deep yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw
Control (no amino-acid)	Yellow	Yellow	Yellow	Yellow	No colour	Straw	No colour	Straw	No colour	Straw	No colour	Straw

Reaction in butanol phase with excess alkali. Although the depth of colour even in dilute solutions was quite satisfactory when the reaction was carried out at pH 11–12 followed by the addition of 2N-NaOH, duplicate values did not show sufficiently close agreement when the reaction was examined quantitatively in the Spekker Absorptiometer (Adam Hilger) under these conditions. After various additional trial experiments it was found that replication of tests could be achieved by allowing the reaction to proceed slowly in the butanol phase, excess alkali being added initially. Under the conditions described below in *Procedure*, it was found by titration that about 5% of the excess alkali in the reaction mixture was in the butanol phase. Since thyroxine is completely transferred to the butanol layer in aqueous alkali-butanol mixtures, this procedure also permitted a maximum reaction to take place while enhancing the specificity. The solution of the diazo compound as well as that of the dye possesses marked stability at high pH. After the addition of the alkaline diazo solution, the reaction can be interrupted at any stage by simply stoppering the tubes, and then started again by allowing access to the atmosphere. Neither light nor temperature has a deteriorating effect upon the reaction provided the pH is maintained at a high level. Thus, after the diazonium hydroxide was added in one test, the tubes were placed in a boiling water bath for 0·5 hr., but neither the control nor thyroxine tubes showed any apparent change. However, on standing overnight at room temperature, the normal reaction took place in the thyroxine tube. On the other hand, elevating the temperature during the reaction by placing the tubes in a thermostat at 25° reduced the time for full colour development. Continual gentle agitation, while not essential, was found to assist the reaction by equalizing the fall in pH in the butanol layer. Violent shaking, on the other hand, facilitated diffusion of alkali from the aqueous phase, so that the pH did not fall to a level for colour development.

The mechanism of the reaction probably depends on the *slow* absorption of CO_2 from the atmosphere by the NaOH contained in the butanol phase, as no colour develops when the tubes are stoppered, or when CO_2-free air is passed over the butanol surface. Various attempts were made to speed up the reaction by the addition of minimal amounts of dilute acid, buffer solutions or salts, or by passing CO_2 over the butanol surface; however, neither duplicates nor colour quality were as satisfactory as by the slow process. The reaction was also tried unsuccessfully in *n*-butanol with all solutions prepared in this solvent, the diazotization being carried out with *n*-butyl nitrite.

Specificity. Table 3 gives a list of phenols, diiodophenols and amino-acids tested under the procedure ultimately adopted and described below. Thyroxine and thyroxamine produce about the same depth of colour, while that from diiodothyronine is very much weaker. Thyronine behaves like diiodotyrosine and tyrosine. The peptide, glutamylthyroxine, also gives a negative reaction under these conditions; on the other hand, thyroxyldiiodotyrosine, owing to its preference for the butanol phase, gives a stronger reaction, at equivalent dilution, than thyroxine.

Table 3. *Reactions between diazotized N¹-diethylsulphanilamide and various hydroxy compounds and amino-acids, compared with thyroxine*

Compound	Conc. (%)	Colour produced	
		Immediately	After 24 hr.
Thyroxine	0·005	No colour	Light purple
Thyroxamine	0·005	No colour	Light purple
3:5-Diiodothyronine	0·005	No colour	Pale pink
Thyronine	0·005	No colour	Straw
3:5-Diiodotyrosine	0·02	No colour	Straw
Tyrosine	0·02	No colour	Straw
Control	—	No colour	Straw
Glutamylthyroxine	0·005	No colour	Very light yellow
Thyroxyldiiodotyrosine	0·005	No colour	Purple
3:5-Diiodotyrosine methyl ester	0·005	No colour	Straw
3:5-Diiodo-4-hydroxybenzoic acid	0·005	Straw	Yellow
3:5-Diiodo-4-hydroxybenzaldehyde	0·005	No colour	Straw
*3:5-Diiodo-4-hydroxyphenyllactic acid	0·02	No colour	Yellow
3:5-Diiodo-*p*-cresol	0·005	Pink	Deep red
p-Cresol	0·02	No colour	Deep pink
p-Hydroxyphenyllactic acid	0·02	No colour	Yellow
p-Hydroxyphenylpyruvic acid	0·02	No colour	Yellow
p-Hydroxybenzoic acid	0·02	No colour	Yellow
Salicylic acid	0·02	No colour	Yellow
D-Glutamic acid	0·02	No colour	Light yellow
DL-β-Phenylalanine	0·02	No colour	Light yellow
L-Histidine	0·02	Yellow	Deep yellow
L-Hydroxyproline	0·02	No colour	Light yellow
L-Tryptophan	0·02	No colour	Yellow
Glycine	0·02	No colour	Light yellow

* Saul & Trikojus (1948) reported the value of diazotized *N*¹-diethylsulphanilamide in enabling them to follow the course of the conversion of 3:5-diiodo-4-hydroxyphenyllactic acid into the corresponding analogue of thyroxine.

Both components of this dipeptide presumably engage in reaction with the diazo compound. Of the monocyclic types tested, only *p*-cresol and its diiodo derivative gave a positive result.

It should be pointed out that some of the substances tested have not been shown to occur naturally; others could be removed by procedures which would not affect thyroxine. Thus *p*-cresol and its diiodo derivative are readily soluble in ether, and mixtures of these phenols and thyroxine have been shown after ether treatment to match the colour given by the same amount of thyroxine treated similarly. Complete hydrolysis should eliminate irregularities due to contamination by a peptide such as thyroxyldiiodotyrosine. This would also be necessary in a method depending upon the estimation of the iodine content of a butanolic extract.

METHOD

Reagents

Preparation of N^1-diethylsulphanilamide. 4-Acetamidobenzenesulphonyl chloride (40 g.) was dissolved in acetone (200 ml.) and added to a cold aqueous solution of diethylamine, prepared from diethylamine hydrochloride (44 g.) and 2N-NaOH (200 ml.). The mixture was chilled, powdered $NaHCO_3$ (16 g.) added and the reaction allowed to proceed at room temperature for some hours with shaking at frequent intervals. Acetone was removed on the water bath under reduced pressure, the residue was chilled and water (200 ml.) added. The precipitate of N^1-diethyl-4-acetamidobenzenesulphonamide which separated was hydrolyzed by boiling for a few minutes with 6N-HCl (40 ml.). Following the addition of water, heating was continued for a further few minutes. The hot filtered solution was cooled and made alkaline with 2·5N-NaOH. The resulting precipitate was washed with water (crude yield, 32 g.) and recrystallized, first, from 60% (v/v) ethanol with the aid of charcoal, and then from a mixture of ethanol and light petroleum (b.p. 60–80°). It separated as colourless prisms, m.p. 105–106°. (Mangini (1940) records 105–106°.) Found: N, 12·5%. (Calc. for $C_{10}H_{16}O_2N_2S$: N, 12·3%.)

Diazotization. Preliminary tests showed that the large excess of $NaNO_2$ used by Komant was not necessary for the production of a stable solution of the diazonium salt of this amine. Accordingly, the diazotization was carried out as follows: the amine (1·14 g., 0·005 mol.) was dissolved in 10N-HCl (9 ml.) and the solution diluted to 100 ml. with distilled water. 5 ml. of this solution was diazotized at 0° with 5 ml. of 4·5% (w/v) $NaNO_2$ (A.R.); ice-cold water (20 ml.) was then added and the resulting solution used after 10 min. Reduction of the quantity of nitrite did not affect the rate of colour development with thyroxine, while the control was somewhat paler.

n-Butanol. This was purified by distillation over solid NaOH; the fraction boiling at 116° was used in this work.

Procedure

The procedure ultimately used for quantitative tests was as follows: thyroxine (2 ml. of a solution containing 1–5 mg./100 ml. of 0·1N-NaOH) was placed in a glass-stoppered round-bottomed Pyrex centrifuge tube of approx. 15 ml. capacity, followed by *n*-butanol (2 ml.) and then 2N-NaOH (1 ml.). The contents were then thoroughly mixed by shaking the tube sideways (liquid should not pass to the neck of the tube). An alkaline solution of the diazo derivative of N^1-diethylsulphanilamide (3 ml., prepared by adding 1 ml. of 2N-NaOH to 2 ml. of the solution of the diazonium chloride was then added, the tube stoppered and the contents well shaken for 15 sec. After standing for 5 min. the stopper was removed and the tube gently agitated in a rack for 24 hr. in a water thermostat maintained at 25°. The rack, the base of which was suspended at a distance of 17 cm., was swung by a geared electric motor about 60 times/min. through approx. 3 cm. (the dimensions given are not critical). The control tube contained 2 ml. of 0·1N-NaOH plus reagents. After the completion of the reaction, the tubes were stoppered and centrifuged for 30 min. at 2500 r.p.m. The butanol phase was then transferred as far as possible with a Pasteur pipette to a small dry tube. To 0·75 ml. of this solution 0·25 ml. N-ethanolic NaOH was then added and the tubes containing the mixed solutions stoppered pending examination in the absorptiometer using Ilford spectrum green filters. Tests showed that almost identical readings could be obtained after a further 24 hr., providing precautions were taken to prevent evaporation of solvent and access of CO_2. (Note. If ethanolic NaOH is not added, the readings are irregular due to the development of a turbidity. Other measures to counteract this, such as more prolonged centrifuging, temperature stabilization or desiccation with anhydrous Na_2SO_4, were ineffective.)

Absorption characteristics. Fig. 1 shows the result of an investigation of the spectral transmittance of the solution of the dye in *n*-butanol (plus ethanolic NaOH). Comparison was with a control solution prepared under identical conditions, thyroxine only being excluded. The instrument used, the Coleman Spectrophotometer, was kindly made available by Mr A. T. Dick (Animal Health Laboratory, Council for Scientific and Industrial Research, Melbourne). Maximum absorption occurred at approx. 540 mμ. at which point the blank control transmitted 93·6% compared with pure *n*-butanol.

Standard curve. A typical calibration curve of the colour intensity produced by varying concentrations of pure thyroxine is illustrated in Fig. 2. Within the range 1–4 mg./100 ml. consistent results have been obtained provided fresh solutions of thyroxine were used for the standardization. In applying the method to the estimation of thyroxine in biological material, such as thyroid powder, some difficulty was experienced in obtaining suitable butanol extracts of the thyroxine fraction prior to assay. This has now been overcome by preliminary treatment

with ether before extracting the acid-insoluble thyroxine precipitate with butanol or by forcing the thyroxine into an aqueous alkaline phase from a

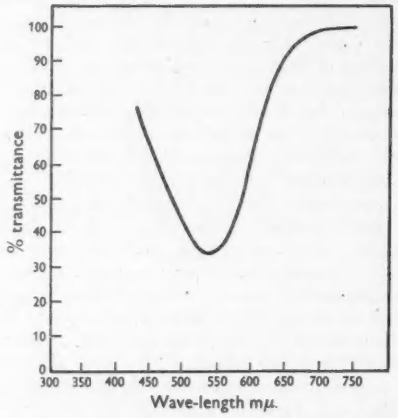

Fig. 1. Spectral transmittance of the colour produced by the reaction of thyroxine (0·01 %) and diazotized N^1-diethylsulphanilamide. Coleman spectrophotometer.

butanol extract by the addition of ether or, preferably, light petroleum. Re-extraction of the aqueous phase with butanol has provided a colourless solution suitable for colorimetric analysis. A report of the results obtained in these studies will form the basis of a further communication.

Note added 10 *October* 1947.

Since this paper was submitted for publication we have had access to a preliminary report by Moser (1947), in which he has shown that by the addition of alkali to the reaction product, Komant's reagent may be used for the rapid estimation of thyroxine in pure aqueous solutions. Our observations indicate that N^1-diethylsulphanilamide is a more stable reagent for this purpose and that the procedure outlined by us greatly enhances the specificity of the reaction.

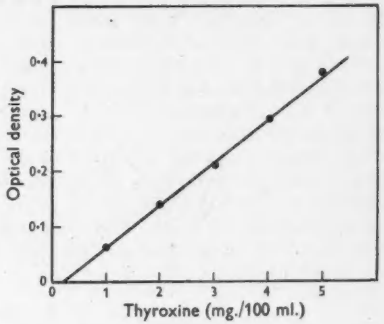

Fig. 2. Calibration curve for the reaction: thyroxine + diazotized N^1-diethylsulphanilamide. Hilger Spekker Absorptiometer with Ilford spectrum green filters.

SUMMARY

A colour test for thyroxine is described based upon its reaction with diazotized N^1-diethylsulphanilamide in alkaline solution. The conditions of the reaction have been studied and a procedure has been suggested whereby thyroxine can be colorimetrically estimated.

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